Studies of serum amyloid P component (SAP) and antinuclear autoimmunity

Julian D Gillmore

Doctor of Philosophy
The University of London

Centre for Amyloidosis and Acute Phase Proteins
Department of Medicine
Royal Free and University College Medical School
Royal Free Campus, London NW3 2PF

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

The plasma pentraxin proteins serum amyloid P component (SAP) and C-reactive protein (CRP) bind specifically to nuclear autoantigens. There is a blunted acute phase response of human CRP in systemic lupus erythematosus (SLE), and of mouse SAP in NZB/W murine SLE. SAP deficiency in (129/Sv x C57BL/6)F_2 mice is associated with spontaneous antinuclear autoimmunity. The pentraxins may thus function in preventing autoimmunity.

Pure line C57BL/6 SAP knockout mice, studied here for the first time, spontaneously developed broad spectrum anti-nuclear autoimmunity resembling human SLE, and, females in particular had proliferative immune complex glomerulonephritis but without proteinuria or renal failure. Mice hemizygous for the SAP gene deletion had an intermediate autoimmune phenotype. Injected apoptotic cells and isolated chromatin were more immunogenic in SAP-/- than in wild-type mice. Extrinsic chromatin was catabolised predominantly in hepatocytes, Kupffer's cells and renal parenchymal cells. Plasma clearance of long chromatin and core particles was marginally slower in SAP-/- mice, with significantly greater splenic uptake of nucleosome core particles, which may have immunological significance. SAP bound to apoptotic cells but had no effect on their phagocytosis in mice *in vivo*, or by human macrophages *in vitro*. CRP also bound to apoptotic cells but did not compete with SAP, indicating recognition of different ligands.

In contrast, to the C57BL/6 strain, pure line 129/Sv SAP knockout mice did not produce autoantibodies. Formation of antinuclear autoantibodies is thus markedly strain dependent. Interestingly, transgenic expression of human SAP in the C57BL/6 SAP knockouts did not abrogate the autoimmune phenotype. Transgenic reconstitution of the knockouts with mouse SAP is currently in progress to confirm that autoimmunity is caused by SAP deficiency and not by 129/Sv genes transferred into the C57BL/6 background during homologous recombination, or by disruption of loci associated with murine SLE that are close to the mouse SAP gene.

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ETHICAL APPROVAL

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ABBREVIATIONS

Abbreviations used in the text are listed alphabetically or are defined when they first appear in the text

Å	Angstrom
ANA	Antinuclear antibodies
APR	Acute phase response
APMoS	Acute phase mouse serum
bp	Base pairs of nucleic acid
BSA	Bovine serum albumin
°C	Degrees Celsius
CFA	Complete Freund's adjuvant
CPM	Counts per minute
CCPM	Corrected counts per minute
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EBV	Epstein-Barr virus
EDTA	Ethylene diaminotetra acetic acid
ENA	Extractable nuclear antigens
EtBr	Ethidium bromide
h	Hours
hSAP	Human SAP
^{125}I	Iodine 125

ICFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-6	Interleukin-6
IP	Intraperitoneal
IV	Intravenous
kb	Kilobases of DNA
kDa	Kilodaltons
LPS	Lipopolysaccharide
MBq	Megabecquerel
min	Minutes
mRNA	Messenger RNA
mSAP	Mouse SAP
NBS	N-bromosuccinimide
OD	Optical density
PBS	Phosphate buffered saline
PCh	Phosphocholine
PCR	Polymerase chain reaction
PE	Phosphoethanolamine
SAP	Serum amyloid P component
SAP+/+	Wild-type mouse with regard to SAP genes
SAP+/-	Mouse hemizygous for targeted deletion of SAP gene
SAP-/-	Mouse homozygous for targeted deletion of SAP gene

SAP-/-, hSAP Tg	"knock-out" SAP-deficient mouse transgenic for human SAP
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
ssDNA	Single stranded DNA
TC	Tyramine cellobiose
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
wk	Week
WT	Wild-type
w/v	Weight for volume

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Chapter 1 - Introduction

HISTORICAL BACKGROUND

Serum amyloid P component (SAP) and C-reactive protein (CRP), the classical acute phase reactant in humans, are normal plasma proteins of hepatic origin, which are members of the pentraxin family of proteins. Although much is known of their structure and properties, the physiological functions of these molecules have yet to be determined. They are, however, highly conserved throughout vertebrate evolution and no protein polymorphism or deficiency of human SAP or CRP have yet been described suggesting that they have important functions. The plasma pentraxins all exhibit calcium-dependent ligand binding that is likely to be of physiological and pathophysiological importance.

Amyloid P component (AP) is a glycoprotein derived from, and identical to, the normal circulating plasma glycoprotein, SAP [1-3]. AP is present in all amyloid deposits in all types of amyloidosis in which it has been sought, both in man and in animals [4,5]. The name P-component was coined by Cathcart *et al* when they discovered, using immunohistochemical techniques, that this protein constituent of amyloid deposits was related to a normal plasma protein [6-8]. Bladen *et al* independently discovered P-component in a negative staining electron microscopic study of amyloid extracts, but they interpreted the characteristic pentagonal molecular structure of SAP as the subunit of aggregated SAP rods which they

believed were the bulk of amyloid deposits [9]. In fact, amyloid deposits are composed largely of amyloid fibrils, derived from a range of different precursor proteins in different forms of the disease, whilst AP is a minor constituent associated with them by virtue of its capacity for calcium-dependent ligand binding to specific determinants shared by all types of amyloid fibrils [4]. SAP was independently isolated and characterised by Haupt *et al* [10] who named it 9.5S α-glycoprotein and Binnette *et al* [11,12] who subsequently demonstrated the identity of this molecule with the pentagonal unit of Bladen *et al* and the P-component of Cathcart *et al.* Further confusion occurred however, when SAP contaminating isolates of the C1 component of complement was mistakenly identified as a fourth subcomponent of C1 and assigned the name C1t [13,14]. Although it was recognised that the putative C1t was SAP [15] and that its N-terminal amino acid sequence and electron microscopic appearance resembled the pentraxin CRP, it was only when the calcium-dependent binding of SAP to agarose was described that the mechanism underlying co-isolation of SAP with authentic C1 subcomponents was appreciated [16,17].

CRP was the first protein to be discovered which behaves as an acute phase reactant, displaying rapid and pronounced rise of its serum concentration in response to infection or tissue injury [18,19]. CRP was initially discovered in 1930 by Tillett and Francis in the serum of patients with acute pneumococcal pneumonia [20]. It was so named because it bound to and precipitated the somatic C-polysaccharide of the pneumococcal cell wall. CRP was subsequently shown to have

calcium-dependent binding specificity for phosphocholine (PC), a constituent of many bacterial and fungal polysaccharides and of most biological cell membranes [21].

PENTRAXINS

SAP and CRP are members of the pentraxin family of proteins, a superfamily of calcium-dependent ligand binding and lectin (carbohydrate binding) proteins. The name pentraxin, coined by Osmand *et al* [22] and derived from the Greek words for five (penta) berries (ragos) reflects the arrangement of the globular subunits of SAP and CRP in a disc-like configuration with cyclic pentameric symmetry, as originally seen by electron microscopy.

Closely related plasma proteins sharing similar sequences, tertiary folds, subunit arrangement and functional properties are present in all vertebrates in which they have been sought, and even in some invertebrates, the most evolutionarily distant of which is the horseshoe crab, *Limulus polyphemus* [23,24]. Several more distantly related proteins with subunits comprising a pentraxin homology domain and an unrelated sequence have lately been identified. These so called "long" pentraxins include PTX3 [25,26], which is induced in hepatocytes, endothelial cells and fibroblasts by IL-1β and TNF-α; several neuronal pentraxins including neuronal activity-regulated pentraxin (NARP) which is the major protein expressed by rat cerebral neurons in relation to physiological neuronal stimulation [27,28]; apexin, a

50 kDa calcium-dependent binding protein of the sperm acrosome [29,30] and a protein in the amphibian, *Xenopus laevis,* KL-PXN1 [31]. Neither the classical nor the long pentraxins show significant sequence homology with other proteins.

SAP AND CRP: GENE AND PROTEIN STRUCTURE

Gene Structure

Pentraxin gene organisation

Human SAP and CRP have a high degree of homology at the amino acid and nucleotide levels. Both leader sequences are hydrophobic and share extensive amino acid sequence homology with a wide range of signal peptides reported for other secreted products. The overall amino acid identity of the mature product is 51%, and 66% when conservative substitutions are considered. The overall nucleotide identity is 59%. The level of homology between SAP and CRP however, is not constant throughout their sequences as a region of limited conservation is bound by two highly conserved regions. Although different levels of homology between different regions may reflect portions of the molecule having similar and dissimilar physiological functions, they do not correspond to separate exons as is often found in the gene structure of other proteins with distinct domains [32].

Human SAP and CRP

The human SAP gene has a relatively simple structure consisting of two exons separated by a 115 bp intron. The transcript is approximately 1.1 kb in size [33].

The first exon contains the mRNA 5'-untranslated region, a sequence encoding the leader peptide and the first two amino acids of the mature protein. The second exon contains the sequence encoding the remaining 202 amino acids and the 3'-untranslated region. The CRP gene (2.5 kb) is similar, except that the intron is considerably larger (278 bp). It contains several interesting features including an unusually long 3'-untranslated region (1.2 kb), three heat-shock consensus sequences in the 5'-untranslated region and a $(GT)_n$ region in the intron that is, in theory, capable of adopting a Z-DNA form and may play a role in chromatin activation [34]. Recently, a G to C polymorphism at nucleotide 1059 within exon 2 of the human CRP gene was reported, but this encodes a silent change at the amino acid level. The frequency of the 1059C allele was 0.109 in the Caucasian population [35].

Murine SAP and CRP

The gene structures of mouse SAP and CRP are similar to the human genes. The SAP transcript of 1065 nucleotides comprises a first exon of 206 nucleotides containing the 5'-untranslated region, the sequence encoding the leader peptide, the first two amino acid residues and the first base of the triplet encoding the third amino acid residue of the mature SAP protein [36-38]. A single 110 bp intron precedes a 749 nucleotide second exon that encodes the bulk of the mature protein and the 141 bp 3'-untranslated region. The 139 bp 5'-untranslated region is larger than its human counterpart (96 nucleotides). As with the human SAP gene, the murine SAP 5'-untranslated region lacks the heat-shock consensus sequences

described in the 5'-untranslated regions of the human and rabbit CRP mRNAs. The 141 bp 3'-untranslated region of the mouse SAP mRNA is 14 residues shorter than that of human SAP. Both SAP 3'-untranslated regions are an order of magnitude shorter than those of the human and rabbit CRP 3'-untranslated regions. There has been less published work on the murine CRP gene but it has been shown to be 1.9 kb in length and have regions that are highly homologous to the human CRP gene including a heat-shock consensus element in the 5'-untranslated region [39].

Chromosomal mapping of pentraxin genes

The gross structural similarities and amino acid sequence homology between SAP and CRP suggest that they are the products of an ancestral gene-duplication event. This hypothesis is strengthened by the mapping of a single copy of the mouse SAP gene to the distal portion of chromosome 1, a region that is syntenic with the human portion of chromosome 1 containing band q2.1, to which both SAP and CRP have been mapped [40-42]. In both species this evolutionarily conserved part of the genome contains a considerable number of immunologically important loci, including genes encoding Fc receptors [43,44].

Protein Structure

Subunit arrangement

Each molecule of human SAP consists of five globular subunits arranged in a disc-like configuration with cyclic pentameric symmetry, forming a ring 95 Å in diameter with a substantial hole in the centre [2,22]. Each identical protomer of

SAP contains 204 amino acids in a single chain with an intrachain disulphide bond between Cys36 and Cys95 [32]. There is a single *N*-glycosylation site at Asn32 and this bears a single typical complex biantennary oligosaccharide chain which does not show the microheterogeneity usually characteristic of glycoproteins [4]. Electrospray mass spectrophotometric analysis reveals a single protomer mass of 25,462 Da, corresponding precisely to the mass predicted from the known amino acid sequence and the known glycan.

The structure of CRP is very similar to that of SAP and consists of five identical non-covalently associated 206 residue protomers arranged symmetrically around a central pore [45,46]. However, unlike SAP, CRP is not glycosylated. The overall dimensions of the CRP pentamer are about 102 Å outside diameter with a central pore diameter of 30 Å and a protomer diameter of 36 Å.

Electron microscopic appearance

The appearances of negatively stained preparations of human CRP and SAP under the electron microscope as annular discs with cyclic pentameric symmetry are highly characteristic [9,22,47]. CRP and SAP from different species vary in the tendency of the basic pentameric discs to associate with each other to form two-disc, 10 subunit structures and subsequently for these decameric molecules to stack up during preparation for electron microscopy [24,47]. Human CRP is almost always seen as single pentameric discs with only occasional pairs and no tendency to stack,

whereas human SAP is always seen as pairs of discs with a variable but always high propensity to stacking. Mouse SAP behaves very similarly to human SAP.

While electron microscopic appearances are important, they are susceptible to *in vitro* artefact in terms of disc pairing and stack formation which do not necessarily reflect the real structure of the native SAP and CRP molecules *in vivo*. For example, the decameric structure was assumed to be the normal physiological configuration of the SAP molecule but recently SAP was shown to exist as a pentamer in whole serum [48]. Electrostatic forces are clearly important in decamerisation of two pentamers, as rapid, complete and reversible decamer-pentamer transitions are achieved by transfer between different buffers [5,49].

Crystal structures of SAP and CRP

The crystal structures of both SAP and CRP have been determined at high resolution [32,45,46]. The SAP protomer is composed of a sandwich of two large antiparallel β-sheets, with a core of hydrophobic side chains and the single α-helix of SAP folding against one face of this sandwich. The fold of the SAP protomer is that of a jellyroll and is related to the folds of the other members of the lectin fold superfamily [50], such as concanavalin A and pea lectin. This relationship is not apparent at the sequence level and other features, such as the location of the metal binding sites, are not conserved. The calcium-dependent ligand binding site of SAP is situated on the opposite face to that of the α-helix and is formed mainly by polar residues from a long irregular loop, co-ordinating two calcium ions 4 Å apart.

Extensive contacts are formed between the protomers and their β-sheets are roughly perpendicular to the five-fold symmetry axis of the pentamer, which has a polarity defined by 5 α-helices on one face (A face) and 5 calcium dependent ligand binding sites on the other (B) face. The pentamer is thus a strongly dipolar molecule, with a negative A face and a positive B face. The glycosylation site, Asn 32, is located on the A face at the periphery of the pentameric ring. The oligosaccharide chains are disordered in the crystals and although their orientation is unknown, it is likely that they are extended [51].

Like that of SAP, the CRP protomer is composed of a sandwich of two antiparallel β-sheets against which is folded a long α-helix. On the A face of each subunit there is a marked furrow which defines a region 24 Å long, 7.5 Å deep and 12.4 Å wide. The furrows of the pentamer follow the curvature of the subunits and come close together as they enter the central pore. The outer part of the furrow is positively charged and the inner part, which terminates half way through the pentamer pore at residue Asp112, provides a ring of negative charge lining the pore. On the opposite face of the protomer to that of the α-helix is the calcium-dependent ligand binding site of CRP [45]. Thus the CRP molecule, like that of SAP, has two faces. However, unlike SAP, where all protomers are on the same plane, each subunit in CRP is rotated by 22° towards the pentameric fivefold axis. As a result of this rotation the α-helices lie closer to the fivefold axis while the bound calcium ions are carried away from it.

SAP and CRP: LIGAND BINDING PROPERTIES

SAP Ligands

SAP shows calcium-dependent binding to a wide variety of ligands. The capacity of SAP for specific calcium-dependent ligand binding was first described with respect to agarose, although the affinity of SAP for polyanions and metal ions, including barium, strontium, cobalt, zinc and nickel had already been observed by Haupt *et al* in their work on the 9.5S α1-glycoprotein [10]. Since the original demonstration of calcium-dependent agarose binding [16], SAP has been reported to bind to glycosaminoglycans especially heparin, heparan sulphate and dermatan sulphate, to mannose terminated glycans and to glycans with preterminal galactose residues [52-55]. The best characterised carbohydrate ligand of SAP is the pyruvate acetal of galactose that occurs in agarose and a synthetic monosaccharide form methyl 4,6-O-(1-carboxyethylidene)-β-D-galactopyranoside (MOβDG) used experimentally to determine ligand binding properties of SAP *in vitro* [56]. SAP binds even more avidly to phosphoethanolamine (PE) and calcium-dependent affinity chromatography on PE covalently immobilised via its amino group is the method of choice for isolation of SAP from serum or amyloid extracts. Unlike CRP, SAP does not bind to phosphocholine whereas both bind to PE and these differing interactions allow isolation of these pentraxins from whole serum [57-59].

SAP also binds avidly to DNA and chromatin and is the single protein from whole serum that shows calcium-dependent binding to these ligands at physiological pH

and ionic strength [60,61]. In addition to its avid binding to chromatin, SAP also binds *in vitro* to nucleoli in intact nuclei of cells permeabilised by fixation but not to any other nuclear or cytoplasmic structures [62]. SAP binds avidly to all types of amyloid fibrils *in vivo* [63-65] and to fibrils extracted *ex vivo* [66]. Although this interaction may involve the glycosaminoglycans (GAGs) that are universally associated with amyloid fibrils formed *in vivo* [67], SAP also binds to fibrils formed from pure proteins or peptides *in vitro* [68].

SAP binds to a variety of bacterial pathogens including *Streptococcus pyogenes, Neisseria meningitidis* and *Escherichia coli* although the specific ligand for SAP expressed by these organisms is unknown [69]. SAP binds to Gram-negative bacterial lipopolysaccharide (LPS) [70], although this is not the only ligand for SAP on Gram-negative bacteria since there is marked binding of SAP to an LPS-deficient mutant of *Neisseria meningitides* [69].

It has been shown that closely associated pairs of SAP molecules show specific calcium-dependent binding to fibronectin, C4-binding protein and to a lesser extent IgM [71]. Aggregated SAP shows calcium-independent binding to C1q and can activate the classical complement pathway *in vitro* [72], but non-aggregated SAP shows no significant interaction with any of these molecules. Despite conflicting reports, it has recently been shown very rigorously that SAP exists exclusively as single pentamers in whole undiluted normal serum and is not complexed with any macromolecular ligand within its physiological milieu [48].

Binding of SAP to a wide range of ligands has been reported in the literature including CRP, phosphocholine, aggregated IgG, soluble immune complexes and type IV collagen [73-76]. Concerns have been expressed that these may not represent true physiological binding reactivities of SAP for several reasons. For example, the calcium-dependent autoaggregation of human SAP removed from serum has to be overcome by the addition of bovine or human serum albumin, at the physiological concentration of 4%, into all buffers in which isolated SAP is used for binding studies [5]. In other solvents, including those with lower concentrations of albumin, varying degrees of aggregation occur which modify the binding properties and ligand specificity of SAP [48]. Immobilisation of SAP or its ligands on microtitre plates may cause denaturation of the adherent protein [77] and have unpredictable effects on binding. Also, binding reactions and other biological properties of peptides from the SAP sequence may bear little relationship to the function of the whole molecule [78-82] and since SAP is markedly proteinase resistant in the universally calcium-containing extracellular milieu, it is unlikely that the generation of significant concentrations of such fragments will occur *in vivo*.

CRP Ligands

CRP, like SAP, shows binding to a wide variety of ligands. CRP was first identified by its reactivity with the C-polysaccharide of the pneumococcus [20]. This binding reaction, which is calcium-dependent, results from the specific capacity of CRP to recognise phosphocholine (PCh) residues [21] which are widely distributed in techoic acids, capsular carbohydrates and lipopolysaccharides of bacteria and other

micro-organisms. The presence of PCh has been reported in *Streptococcus pneumoniae* [83], *Haemophilus influenzae* [84], *Pseudomonas aeruginosa, Neisseria meningitides, Neisseria gonorrhoeae* [85], *Proteus morganii* [86] *and Aspergillus fumigatus* [87]. PCh is present in the outer leaflet of most biological membranes as the polar head group of lecithin and sphingomyelin. Initial evidence for binding of CRP to cell membranes was provided by experiments demonstrating the presence of CRP associated with cell membranes at sites of inflammation and tissue necrosis but not in normal cells [88]. It was subsequently shown that CRP could react with emulsions of the PCh-containing phospholipids lecithin and sphingomyelin [89]. Using lecithin liposomes and unilamellar vesicles the requirement of additional submicellar concentrations of lysolecithin for the binding of CRP to the PCh polar head group in lipid bilayers was demonstrated [90]. CRP, like SAP, also binds specifically to PE in a calcium-dependent manner.

CRP binds specifically to small nuclear ribonucleoprotein particles (snRNPs) in intact nuclei of cells permeabilised by fixation but not to any other nuclear structures [62].

Ligand-complexed CRP shows calcium-independent binding to C1q, the first component of the complement cascade, which results in activation of the classical pathway [89,91].

The binding of CRP to FcγRI (CD64) [92] and FcγRIIa (CD32) [93] on phagocytes with low and high affinity has been reported, and more specifically binding of CRP to the R131 polymorphic form of the FcγIIa receptor [94]. However, this was recently shown to be due to contamination of the CRP by IgG and pure CRP did not show any such binding [95]. The conclusion of the authors was that there was no evidence of a specific CRP receptor on human white blood cells.

Molecular Basis of Ligand Binding

SAP

Co-crystallisation of SAP with MOβDG and PE and analysis of the complex with the nucleotide dAMP have shown that an acidic functional group of the ligand (the pyruvate carboxylate group of MOβDG or the phosphate ester moiety of PE and dAMP) bridges the two calcium ions in the ligand binding pocket of each SAP protomer [5,96]. The use of a series of synthetic MOβDG analogues to inhibit the binding of SAP to both amyloid fibrils and high pyruvate agarose demonstrated that the critical determinant of recognition by SAP is integrity of the carboxyethylidene ring [5,56,97].

The avid binding by SAP of macromolecular ligands such as nucleosomal DNA, glycosaminoglycans (GAGs) and amyloid fibrils is likely to be the result of a multi-site interaction involving the B face (bearing the calcium-dependent binding sites) of the SAP pentamer. However, the five fold symmetry of SAP does not match any of its macromolecular ligands, and since co-crystallisation of SAP with

larger biological ligands has yet to be achieved, it is difficult to extrapolate from the few available structures with small synthetic ligands. The binding of SAP to DNA and heparin is calcium-dependent but these ligands may not bind directly to SAP through the two calcium ions and it is impossible to construct a model complex between SAP and dsDNA that involves a direct interaction of the phosphate backbone and the two resident calcium ions [5]. DNA may, therefore, bind in the basic grooves between the protomers of the pentamer, which are of the right size to accommodate a DNA double helix, and the calcium may have an allosteric effect. Although SAP may bind to the GAGs found on amyloid fibrils *in vivo* [67,98], it does interact directly with fibrils formed from pure proteins or peptides *in vitro* [5,68]. SAP may recognise certain features of the amyloid fibrils such as turns between the strands [99], and the spacing between these turns may accommodate the SAP pentamer.

CRP

Ligand binding by CRP, like SAP, is calcium-dependent and on the B face of the protomer, two calcium ions are ligated to side chains and main chain carbonyls of the polypeptide chain at a distance of 4 Å from each other. Crystallographic analysis of CRP and PCh and analysis of the CRP-PCh complex [46] have demonstrated that two of the oxygens of the phosphate group directly co-ordinate with the two CRP-bound calcium ions while the choline group rests within the hydrophobic pocket of the CRP protomer. Mutational studies have confirmed the importance of the hydrophobic pocket of CRP for PCh-binding [100].

The structure and topology of the C1q-binding site have also been investigated by site-directed mutagenesis [101,102]. The mutational data combined with the known structure of the molecule indicate that the C1q-binding site is located on the A face of each protomer at the shallow end of a cleft which extends from the centre of the protomer to its edge at the central pore of the pentamer. The cleft is formed on one side by the α-helix and on the other by parts of the amino and carboxyl termini of the protomer. Residues Asp^{112} and Tyr^{175} are considered to be the C1q contact residues as their substitution with Ala results in significantly reduced avidity for C1q and diminished complement activating ability.

SAP AND CRP: BIOLOGICAL FEATURES

SAP Serum Concentration

Human SAP

Messenger RNA for SAP was initially reported to be exclusively found in hepatocytes [103] although McGeer's group reported recently that SAP mRNA is present in pyramidal neurons of the brain as well as the lungs, heart, arteries, kidneys and spleen [104]. However, adequate tissue and antibody controls were not used in this latter study. The human SAP promoter has been shown to direct expression of linked transgenes exclusively to the liver and this is thought to be under control of elements in the 5' region of the gene [105,106]. Human SAP mRNA can be induced *in vitro* in hepatoma cells by IL-6 but can be down-regulated with IL-1β, an effect that predominates when both cytokines are present [107].

The plasma concentration of human SAP is tightly regulated, being slightly lower in women {mean (SD), 24 (8) mg/l} than men {32 (7) mg/l} [108]. The value remains normal even during massive deposition of SAP into amyloid indicating that although human SAP is not an acute phase reactant, the rate of synthesis and secretion can be upregulated [65,109], the plasma pool of SAP being in equilibrium with the large amyloid-associated pool of SAP. The concentration of SAP in cord sera is much lower at approximately 4 mg/l but rises rapidly during the first weeks of life to reach the lower part of the adult range [110]. In conditions characterised by an acute phase response, the SAP concentration does rise but tends to remain close to the normal range and does not exceed 100 mg/l [110], although patients with macroglobulinaemia may be exceptional in having high levels of SAP in the absence of an acute phase response [111,112]. Among patients with renal insufficiency, SAP concentration is significantly elevated although there is no correlation with serum creatinine or duration of disease [108]. Significantly low concentrations of SAP have only been seen in patients with hepatocellular impairment of sufficient severity to reduce plasma protein synthesis [110,113].

Murine SAP

Murine experiments in the early 1980s suggested that SAP was synthesized, possibly exclusively, in the liver. It was shown that in mice undergoing a vigorous acute phase response the hepatic cytoplasm stained immunospecifically with anti-SAP, particularly in the periportal zone. Administration of colchicine to mice receiving an acute phase stimulus reduced or abolished the rise in SAP. At the same time, the

staining of hepatocytes with anti-SAP became more intense suggesting that the liver was the site of synthesis and that colchicine, by inhibiting secretion of SAP, caused it to be retained within the cells [114,115]. Immuno-electron microscopy showed that SAP was associated with the rough endoplasmic reticulum of hepatocytes.

Murine SAP has several specific characteristics. Firstly, there are marked genetically determined differences in normal serum concentration between different inbred strains. C57BL mice and lines with the same background have concentrations of up to 10 mg/l, whilst DBA/2 mice may have levels as high as 100 mg/l. Other strains such as C3H, BALB/c, CBA, and A/J have intermediate serum concentrations of 20-80 mg/l, but crosses with C57BL mice always produce progeny with low levels [116,117].

Secondly, SAP is a major acute phase reactant in all mouse strains. Hepatic SAP mRNA levels massively increase within two to four hours and the concentration of SAP in the serum starts to rise by about eight hours after an acute physical or chemical injury or inflammatory stimulus and reaches a peak at about 24-48 hours. Background levels of both hepatic mRNA and plasma protein are re-established by 72 hours after a single transient stimulus. There are no differences between male and female mice and acute phase levels are derived from *de novo* synthesis of the protein rather than release of preformed stocks. Mice depleted of T- and B-cells, rendered agranulocytopaenic by irradiation or hypocomplementaemic by cobra venom factor all respond to injections of casein or croton oil with the same SAP

concentration as controls [116]. *In vitro* studies of SAP synthesis in mouse hepatocyte cultures show that SAP mRNA and protein levels increase in response to stimulation with a variety of cytokines such as IL-1, IL-6, TNF-α and TGF-β [118]. The addition of IL-1α caused a tenfold increase and the addition of IL-6 a sevenfold increase; the addition of both cytokines together had an additive effect [107].

Finally, elevation of SAP concentration following stimulation of the acute phase response correlates with amyloid deposition in murine models of this disease [119]. Repeated subcutaneous injections of casein can induce AA amyloidosis in mice [120]. Strains that are more susceptible to this, such as C57BL and CBA, develop persistent elevation of SAP concentration (10-100 times baseline) during administration of casein, which subsequently fall to normal when the injections cease. Strains such as A/J mice that are relatively resistant to amyloid deposition develop only transient elevation of SAP concentration during casein treatment. All strains develop similar elevations in serum amyloid A (SAA) concentration, the precursor protein of the amyloid fibrils, during casein administration. Mice given bovine serum albumin, which also induces elevation of SAA, do not develop amyloidosis and produce only small, transient increases in SAP concentration [119].

SAP Metabolism

The plasma half life of human SAP is 24 hours and is remarkably constant in all individuals who have been studied and in all diseases except amyloidosis [63-65]. In mice the half life of SAP is about 7.5-9.5 hours in all strains studied, regardless of

their state of health and whether they are mounting an acute phase response or have established amyloidosis [63,115,121]. Studies in mice indicate that hepatocytes are the sole site of clearance and catabolism [122]. Persistence of SAP in the circulation is absolutely dependent on the intactness of its oligosaccharide with loss of the terminal sialic acid residues resulting in extremely rapid uptake via the hepatocyte asialoglycoprotein receptor and swift catabolism. Blockade of the asialoglycoprotein receptor in mice, however, does not affect the clearance of SAP indicating that physiological SAP turnover may involve protein rather than carbohydrate recognition [4].

Tissue Amyloid P Component (TAP)

Human

Although SAP had already been extensively investigated in amyloidotic tissue, its presence as a normal constituent of the extracellular matrix in human spleen, liver and kidney was first recognised immunohistochemically in 1978 by Schneider and Loos [123]. Dyck *et al* then characterised the normal tissue AP (TAP) of human glomerular basement membrane and showed that it is confined to the *lamina rara interna* where it is covalently associated with collagen and possibly other matrix constituents [124]. It was found that SAP is also associated with the microfibrillar mantle of elastic fibres throughout the body and that in the skin at least, TAP is non-covalently bound, suggesting that it is not an integral constituent of elastic fibre microfibrils [125-128]. TAP is absent or abnormally distributed in a range of glomerular and elastic tissue disorders although its functional significance remains

unknown [127,129]. Although TAP has not been fully characterised, it is immunochemically identical to SAP and may be derived from circulating SAP, originally produced in the liver. This is consistent with the age-dependent appearance of TAP in the tissues, being undetectable until the third year of life and increasing to approximately adult concentrations by five years [130].

It is possible that the roles of TAP include modulation of the biological properties of the matrix GAGs and/or protection of proteinase sensitive structures (basement membrane, elastic fibres). The density of TAP molecules suggest that they are likely to achieve the close pairing required for recognition and binding of fibronectin and C4-binding protein. There is no evidence however, that C4-binding protein is a normal matrix protein and the distribution of TAP and fibronectin in the tissues is only partly overlapping. The physiological function of TAP therefore remains elusive.

Other species

The existence of TAP in species other than man remains controversial. Leblond *et al* have published a variety of immunohistochemical reports showing TAP in mice and also isolated mouse SAP from the EHS sarcoma, a transplantable tumour with an exceptionally rich connective tissue matrix [131-133]. Pepys' group however has been unable to demonstrate TAP in any species other than man and question the interpretation of the electron micrograph images [5]. It is possible that

the SAP obtained from EHS tissue was derived from the serum acute phase response and not from the tumour matrix.

CRP Serum Concentration

Human CRP

Although messenger RNA for CRP has been reported in extrahepatic tissues such as alveolar macrophages, the hepatocyte is the major source of CRP [134-136]. The principal cytokine mediator of human CRP induction *in vitro* in hepatoma cell lines is IL-6, but some models show a requirement for both IL-6 and either IL-1 or TNF-α [137].

The speed of change and incremental range of CRP concentration are exceptional among all acute phase proteins, apart from SAA. With the ability to increase synthesis by up to 10,000 fold, the distribution of CRP is clearly non-Gaussian and among healthy subjects the median concentration of CRP is only 0.8 mg/l, with an interquartile range of 0.3-1.7 mg/l. Ninety percent of apparently healthy subjects have levels of less than 3 mg/l and 99% of them less than 10 mg/l [138-140]. Serial studies of normal subjects and of monozygotic and dizygotic twins demonstrate that each individual's baseline serum CRP concentration is remarkably constant and is substantially genetically determined [141]. The concentration of CRP in cord sera is extremely low with a median of 0.04 mg/l (range 0.01-0.49) but rises to adult values within a few days [142,143].

Most significant microbial infections are associated with elevated CRP concentration [20,144,145] although uncomplicated viral infections such as the common cold, are frequently associated with a modest CRP response or none at all [146]. Whilst individual infections elicit a variable rise in CRP concentration, the response *per se* exists in all hosts regardless of age [142,143], immune status, or therapeutic intervention [147,148]. Normalisation of the CRP concentration usually corresponds to clinical cure whilst persistently elevated concentration often indicates recurrence or persistence of infection.

With a few exceptions, most inflammatory diseases are associated with elevated CRP concentration which, when monitored serially, reflect the extent and activity of the condition. For example, in rheumatoid arthritis CRP concentration is predictive of future progression of bone erosion and joint damage [149,150], and in Crohn's disease or systemic vasculitis elevated CRP concentration frequently indicates active pathology that may be difficult to detect clinically [151-153]. Many malignancies are associated with an elevated CRP concentration and it has been suggested that in certain conditions such as myeloma [154] and non-Hodgkins lymphoma [155] the degree correlates with tumour burden and prognosis.

There are a few conditions in which there is characteristically, an inappropriate CRP response to autologous tissue damage despite unequivocal evidence of active inflammation. These include the prototypic systemic autoimmune disease systemic lupus erythematosus (SLE) [156,157], ulcerative colitis [151,152] and

dermatomyositis [158]. The mechanism behind this phenomenon is not known, but *in vivo* turnover studies with ^{125}I-labelled CRP have shown no increased catabolism or consumption of CRP in patients with SLE compared to normal controls, indicating a reduced rate of synthesis [159]. Interestingly, patients with these conditions appear to mount an appropriate CRP response to intercurrent infection [156,160,161].

It has been recognised for some time that CRP concentration rises following a myocardial infarction [162,163], and that complications and survival correlate strongly with CRP concentration [164-166]. In recent years however, attention has focused on the importance of CRP concentration as a predictor of future cardiovascular events in normal healthy individuals [167-171]. Large case controlled studies have confirmed that in both men and women the finding of a CRP concentration of greater than 3 mg/l is associated with a two to fivefold increase in risk of future myocardial infarction, cerebrovascular accident or peripheral vascular disease [172].

Murine CRP

In mice, CRP is present in serum at concentrations of less than 2 μg/ml and does not increase markedly during the acute phase response [173,174]. Despite attempts to stimulate CRP production by subcutaneous or intraperitoneal injection of different doses of LPS, croton oil in liquid paraffin, and Freund's complete adjuvant into CBA, C57/BL and BALB/c mice, the serum concentration did not exceed 2 μg/ml.

CRP Metabolism

The plasma half life of human CRP is 19 hours, and is remarkably constant in all individuals in which it has been studied including those with a wide range of inflammatory and neoplastic diseases [159]. The identical turnover profiles of CRP among patients with SLE compared to healthy controls and patients with acute infections indicate that the failure of patients with SLE to mount a marked serum acute phase response of CRP to autologous inflammation and tissue damage is due to failure of CRP production rather than accelerated clearance. The *in vivo* turnover of serum CRP in rabbits is remarkably constant and is unaffected by the intravascular presence of even macromolecular ligands to which CRP binds, such as apoB-containing lipoproteins [175]. Thus, formation of CRP-ligand complexes in the circulation does not accelerate CRP clearance, which apparently proceeds at a constant maximum rate via a mechanism which is not saturable.

Although there are many reports of the variable presence of immunohistochemically detectable CRP in inflammatory and/or necrotic tissue lesions [88,176,177], it has not been clear whether significant CRP is generally deposited at sites of inflammation. More recent studies have found that CRP is deposited in most human atherosclerotic lesions [178,179], and in all human acute myocardial infarcts [180].

SAP AND CRP: PHYSIOLOGICAL AND PATHOLOGICAL ROLES

Pentraxins and Host Defence

Host Defence against infection

The mammalian body is susceptible to a wide variety of pathogens which differ greatly in their lifestyles and host specificity, requiring a diverse set of defensive responses from the host immune system. The immune system of vertebrates has been conceptually divided into two parts, innate and acquired immunity. There are many distinguishing features of the two systems but the essential difference is genetic. The recognition proteins of the innate system are encoded in the germline, having evolved in invertebrates for the purpose of host defence against infection. Innate immunity exhibits rapid response kinetics but lacks memory. It also has the drawbacks of being able to recognise only the relatively few microbial structures that are highly conserved and being unable to evolve as rapidly as do micro-organisms. The receptors of acquired immunity, which are the antigen receptors of T and B lymphocytes, have overcome these problems by not being encoded in the germline. Rather, they are the products of somatically rearranged elements, the V, D and J segments of T-cell receptor and antibody genes. This remarkable capability occurred suddenly with the evolution of vertebrates approximately 400 million years ago [181] and created an enormous repertoire of antigen binding structures. Acquired immunity has slow response kinetics but the ability to remember.

Innate immunity

The first phase of host defence is innate immunity, which is responsible for detecting and destroying most of the micro-organisms that are encountered by a healthy individual. Organisms that penetrate the epithelial surfaces of the body are faced with two immediate lines of defence. First, they are subject to humoral attack by the alternative pathway of complement activation, which is spontaneously active in plasma and can opsonise or destroy many organisms whilst sparing host cells which are protected by complement regulatory proteins. Second, they may be recognised directly and engulfed by phagocytic macrophages and neutrophils, primarily situated in subepithelial connective tissues, that have receptors for common pathogenic components. The other effects of the interaction of phagocytes with pathogens include the secretion of cytokines by phagocytes and the expression of co-stimulatory molecules on the surface of macrophages that allow them to function as professional antigen-presenting cells in the adaptive immune response.

If the micro-organism evades or overwhelms innate defences a subsequent wave of responses involving the activation of a variety of humoral and cell-mediated effector mechanisms are initiated that form the early induced (non-adaptive) response. These responses are triggered by receptors that are non-clonal or of limited diversity and are distinguished from adaptive immunity by their failure to provide immunological memory. Certain such responses are induced by cytokines released by phagocytes in response to microbial infection; for example, induction of production of acute phase proteins which can bind to bacterial surface molecules and activate complement.

Interferons are produced by cells infected with viruses; they slow viral replication, enhance presentation of viral peptides to cytotoxic T cells and activate natural killer (NK) cells. NK cells, CD5 B cells and γ/δ T cells are lymphocytes with receptors of limited diversity that provide early protection from a limited range of pathogens but do not generate lasting immunity. All these mechanisms play an important role in holding infection in control during its early phases while the adaptive response is being generated.

Adaptive immunity to infection

Adaptive immunity occurs when pathogens have evaded non-adaptive mechanisms of host defence and established a focus of infection. Antigens of the pathogen are transported to local lymphoid organ by migrating antigen presenting cells or trapped there by resident cells. Such antigens are processed and each is presented to antigen-specific naïve T cells, the end result of which is production of effector T cells that either leave the lymphoid organ to effect cell-mediated immunity in sites of infection in the tissues or remain in the lymphoid organ and participate in humoral immunity by activating B cells. The type of response that occurs is determined by the differentiation of CD4 T cells into TH1 or TH2 cells, which in turn, is determined in part by the cytokines produced in the early non-adaptive phase. Ideally, the adaptive immune response eliminates the pathogen and provides the host with a state of protective immunity against re-infection with the same pathogen by developing immunological memory.

The role of acute phase proteins in host defence

Since the beginning of the 20th century many studies have demonstrated non-specific resistance to infection that can be enhanced by administration of killed micro-organisms or preparations derived from micro-organisms, such as bacterial endotoxin. In 1967, Raskova demonstrated protection against the adverse effects of LPS by irritation of tissue by phenol or procaine, thereby mimicking 'endotoxin-induced tolerance' [182]. This effect was transient and could be passively transferred with serum. Evidence has since accumulated that acute phase proteins can mediate such non-specific protection against endotoxin as well as against Gram negative bacterial sepsis. Human CRP was reported to opsonise bacteria *in vitro* [183,184] and *in vivo* inhibition of hepatic synthesis of acute phase proteins was shown to increase sensitivity to LPS-induced lethality [185], an effect that was abrogated by pre-activation of acute phase protein synthesis [186].

Acute phase proteins have a wide range of activities that contribute to host defence. They can directly neutralise inflammatory agents, help to minimise the extent of local tissue damage and participate in tissue repair and regeneration. The changes in plasma concentration of individual acute phase reactants are variable. Most are induced between 50% and several fold over normal concentration. In contrast, the concentration of so-called major acute phase proteins, which include SAA and either CRP in humans or SAP in mice, can increase 1000-fold or more. The magnitude and rapidity of their induction following an acute phase stimulus, together with their

short half-lives, suggest an important role for these proteins early in the establishment of host defence.

SAP and host defence

SAP binds to certain bacteria including the important human pathogens *Streptococcus pyogenes* [187], *Neisseria meningitides* and rough variants of *Escherichia coli* [69], suggesting a possible role in host defence. However, Noursadeghi *et al* showed that this binding had a powerful anti-opsonic effect both *in vitro* and *in vivo,* and enabled bacteria to evade neutrophil phagocytosis and display enhanced virulence. They demonstrated that SAP knockout mice survive lethal infection with *Streptococcus pyogenes* and *Escherichia coli* J5, organisms to which SAP binds, and that the susceptibility of SAP deficient animals was fully restored by injection of isolated human SAP. They suggested that the anti-opsonic properties of SAP are critical for its physiological function in handling chromatin and nucleosomes *in vivo* and that bacteria have evolved a mechanism of using this to their advantage.

In contrast to the effects of SAP binding that are harmful to the host, Noursadeghi *et al* also showed that SAP contributes to survival during infections with organisms to which it does not bind, such as *Salmonella typhimurium.* The mechanisms underlying this effect remain unclear.

Lipopolysaccharide (LPS), or endotoxin, is the major component of the outer membrane of Gram-negative bacteria and is a major distinguishing factor between Gram-negative and Gram-positive bacteria. LPS consists of three main structural elements: the O-specific polysaccharide chain, the core region and the lipid A moiety. Although SAP binds to LPS *in vitro* [70,188] and prevents LPS-mediated classical pathway complement activation [189], SAP deficient mice were only marginally more susceptible to lethal LPS challenge than their wild-type counterparts. Furthermore, injection of large amounts of SAP into wild-type mice did not affect sensitivity to LPS, indicating that SAP is not a significant modulator of LPS toxicity *in vivo* [69].

As well as binding to bacteria, SAP also binds to the influenza virus haemagglutinin and inhibits invasion of cells *in vitro* [190]. Horváth *et al* recently reported that preincubation of the influenza A virus with human SAP *in vitro* or intra-nasal administration of human SAP to mice prevents infection *in vivo* [191], suggesting that SAP might play a role in innate immunity to influenza infection. Studies from our laboratory however, in which we compared SAP deficient and wild-type mice following intra-nasal infection with adapted human influenza virus, did not show any consistent difference with respect to the clinical course or outcome, viral replication and anti-viral response. It was concluded that binding of SAP to influenza virus *in vivo* has no significant functional consequences [192].

CRP and host defence

Activation of the classical complement pathway by binding of CRP to phosphocholine and other phosphate ester-containing compounds [91], together with reports that CRP binds to monocytes [92,193] and neutrophils [194] imply a significant role for CRP in host defence. CRP was first shown to effectively prevent mortality from pneumococcal challenges in 1981 by Du Clos' group [195]. They demonstrated that a bolus injection of CRP protected mice from infection with serotypes 3 and 4 of *Streptococcus pneumoniae*, a finding which was later repeated by Volanakis' group [196]. Similar results were obtained by Szalai *et al* with transgenic mice expressing human CRP [197], and the conclusion from these studies was that the opsonic properties of CRP were responsible for its protective effect. Xia *et al* showed that transgenic mice expressing high levels of rabbit CRP were partially protected from a lethal challenge of bacterial LPS compared to littermates in which CRP expression had been suppressed [198]. However, the authors also observed similar protection with challenges from platelet-activating factor (PAF) and a combination of TNF-α and interleukin-1β (IL1β), and showed that the mechanism by which CRP provides protection probably does not involve sequestration of PAF, consistent with the view that CRP plays a significant role in host defence other than by direct opsonisation.

Serum Amyloid P Component in Amyloidosis

SAP is present in all amyloid deposits in all tissues, where its concentration relative to that in the plasma is remarkably high, reflecting the avid calcium-dependent

binding of SAP to amyloid fibrils [66,199,200]. These observations suggested that the association of SAP with amyloid may be related to the pathophysiology of amyloidosis, evidence for which was initially obtained by comparisons of susceptibility, in different mouse strains, to casein induced AA amyloidosis [201]. Whilst there was no difference between these strains in the acute phase response of serum amyloid A protein (SAA) (the amyloid fibril precursor in casein induced AA amyloidosis), there was a marked difference in the SAP response between susceptible and resistant mouse strains, with sustained high levels of SAP in susceptible strains and temporary and modest SAP levels in resistant strains [119]. Similar findings have been reported in other studies [202,203].

The demonstration of a very long half life of SAP in human amyloid deposits of 30 days compared to 24 hours in the circulation [65], coupled with the completely unaltered nature of AP in amyloid deposits compared to plasma SAP [4] and the extreme proteinase resistance of SAP [68,204] was further support for a pathophysiological role of SAP in amyloidosis. The binding of SAP to amyloid fibrils *in vitro* protects them against proteolytic degradation [68] and it is probable that AP serves the same function *in vivo.*

Investigation of effects of human SAP on *in vitro* amyloid fibril formation from soluble precursor proteins is complicated by the inevitable calcium-dependent self-aggregation of SAP. Various authors have suggested that SAP promotes fibrillogenesis, however, these experiments were usually carried out in conditions in

which the SAP autoaggregates [205,206] or in the absence of calcium [207] which is entirely un-physiological. There is thus no compelling, physiologically relevant evidence of any effect of SAP on fibrillogenesis *per se.*

In order to explore further the role of SAP in amyloidogenesis, mice totally lacking SAP by targeted deletion of the SAP gene were created at Hammersmith Hospital [208]. The mice grew, developed and survived apparently normally in standard animal house conditions, were anatomically normal and fertile. However, the induction of AA amyloidosis by repeated casein injection was highly significantly delayed in comparison with SAP-sufficient controls, providing the first unequivocal evidence of a contributory role for SAP in amyloid pathogenesis [208].

Extensive clinical studies with radiolabelled SAP as a specific quantitative *in vivo* tracer for amyloid have demonstrated that amyloid deposits are in a state of dynamic turnover and that reducing or abolishing new amyloid deposition is associated with regression of existing amyloid deposits and frequent clinical benefit [209-211]. Any contribution to accelerated clearance of existing amyloid and/or to retarded deposition would therefore be beneficial and could have a major impact on the morbidity and mortality of the systemic amyloidosis syndromes. In view of the protective effect of human SAP against proteolysis of amyloid fibrils *in vitro*, and delayed amyloidogenesis in SAP deficient mice, inhibition of binding of SAP to amyloid fibrils *in vivo* is a rational and attractive therapeutic target in all forms of amyloid-related disease.

With this objective in mind, a drug discovery program was recently embarked upon. A drug, (R)-1-[6[(R)-2-Carboxy-pyrrolidin-1-yl]-6-hexanoyl]pyrrolidine-2-carboxylic acid, was designed which is a competitive inhibitor of SAP binding to amyloid fibrils. It is a palindromic compound which cross links and dimerises SAP molecules leading to their very rapid clearance from the circulation by the liver. It produces remarkable depletion of circulating SAP and is currently being clinically evaluated in patients with systemic amyloidosis [212].

Pentraxins and Systemic Lupus Erythematosus

Pathogenesis of systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is the prototypic systemic autoimmune disease with clinical features that include glomerulonephritis, rashes, serositis, haemolytic anaemia, thrombocytopaenia, and central nervous system involvement. The characteristic immunological abnormality is the presence of high affinity, somatically mutated IgG autoantibodies directed against ubiquitous intracellular, cell-surface and extracellular autoantigens [213]. The spectrum of intracellular autoantigens includes the proteins and DNA that form chromatin, proteins of the spliceosome complex and the Ro/La small cytoplasmic ribonucleoprotein particle. Autoantibodies to cell-surface antigens include anti-phospholipid antibodies and autoantibodies to red and white blood cells and platelets.

Pathogenesis of SLE - Genetic and environmental factors

Human lupus has an important genetic component as illustrated by 24% concordance of disease between monozygotic twins compared with 2% between dizygotic twins [214]. However, the observation that the second twin does not develop lupus in three-quarters of monozygotic twin pairs also indicates an important role for environmental or stochastic factors.

Genetic factors

A number of linkage studies have mapped genetic loci (each containing multiple genes) that influence the development and expression of SLE in both humans and mice. There are a number of mouse strains in which SLE reproducibly develops in all members of colonies held in standard animal houses worldwide. Thus far, linkage analyses in multiple murine models have detected 31 susceptibility loci distributed among 21 non-overlapping 20 cM genomic intervals, illustrating the complexity of the genetic basis for susceptibility to systemic autoimmunity [215]. However, genomic segments on murine chromosomes 1 (*Sle1)*, 4 (*Sle2)* and 7 (*Sle3)* are associated with disease susceptibility in multiple strain combinations, suggesting that these intervals may contain genes or gene clusters that strongly influence autoimmunity. Further analyses of congenic mouse strains carrying genomic intervals with *Sle1, Sle2* and *Sle3* have provided a detailed characterisation of the component autoimmune phenotype produced by each of these susceptibility genes. *Sle1* mediates the loss of immune tolerance to chromatin and leads to the appearance of high titres of antinuclear autoantibodies (ANAs) and initiation of autoimmunity, and both *Sle2 and Sle3* mediate

polyclonal activation affecting the B cell lineage. In addition *Sle3* mediates the production of ANAs and, in contrast to *Sle1,* can cause the development of severe lupus nephritis [216-218]. A bicongenic strain bearing both *Sle1* and *Sle3* was subsequently shown to exhibit splenomegaly, expanded populations of activated T and B cells, an IgG autoantibody response targeting multiple components of chromatin, and highly penetrant glomerulonephritis [219]. Fine mapping analysis of the genetic location of *Sle1* has demonstrated that three loci within this congenic interval, termed *Sle1a, Sle1b* and *Sle1c,* can independently cause loss of tolerance to chromatin [220].

In humans, both genetic linkage studies and association studies analysing the contribution of numerous candidate genes have been performed [221]. The first human linkage studies in SLE were done by Bias *et al,* and suggested an autosomal dominant mode of inheritance [222]. However, poorly informative markers were used and there was a paucity of pedigree material available for study. More recent approaches have identified particularly strong linkage with the syntenic equivalent of *Sle1* in the mouse, 1q21-44 [223-226]. This region of chromosome 1 contains several interesting candidate genes including the Fc receptors at 1q21-23 which have previously been linked to SLE susceptibility by association and case control studies [227,228]. The Fc receptors for IgG – FcγRI, FcγRII, and FcγRIII are all encoded within this region and bind IgG-containing immune complexes with distinct affinities [229]. Furthermore, FcγRII, and FcγRIII allele distribution has been reported to differ between SLE patients and the normal population [228,230,231]. Other genes of interest within this region of chromosome 1 include SAP and CRP

[232], PARP, TGFβ2 and *ADPRT*, a gene that is involved in apoptosis and cellular proliferation [233].

The genetic basis for SLE is unknown in the vast majority of patients with the condition. However, in a tiny minority of humans with SLE and in certain mouse strains, single gene abnormalities are responsible for determining disease susceptibility. In humans, as well as the Fc receptor genes mentioned above, certain inherited deficiencies of proteins of the classical pathway of complement, are almost invariably associated with the development of SLE [234-236]. Recently, ablation of the *Dnase 1* gene in mice was reported to result in a lupus-like syndrome, and the activity of Dnase 1, the major serum nuclease, was reduced in patients with SLE compared to normal controls [237]. The MRL mouse strain is prone to spontaneous development of lupus; however susceptibility to SLE is dramatically enhanced by the presence of the *lpr* gene, which encodes a mutated fas protein, a cell surface receptor which regulates apoptosis in several cell types including lymphocytes [238]. The *gld* gene, associated with a similar phenotype in mice to *lpr,* was subsequently discovered to encode a mutated fas-ligand [239]. There is strong evidence in both humans and mice with SLE for the participation of the products of genes of the major histocompatibility complex (MHC). Certain polymorphic products of MHC class II genes, influencing the selection of peptides to be presented to T cells, are strong candidates for modifying disease susceptibility [240]. However, there is also evidence that other genes encoded in the region of the MHC

may be important, such as the *C2* and *C4* genes, and the genes for the cytokines TNF-α and lymphotoxin-α [241].

Environmental factors

The very low prevalence of SLE in rural black populations in West Africa compared with a prevalence of up to 1:250 in female African Americans suggests a role for important environmental influences in protection against development of lupus in Africa. It has been speculated that the high incidence of infectious disease, especially malaria, in Africa may protect against development of SLE [242].

The best characterised environmental agent predisposing to the development of SLE is intake of certain drugs, such as hydrallazine and procainamide. However the phenotype of drug-induced lupus is different from the idiopathic disease, with a low prevalence of glomerulonephritis and predominance of anti-histone rather than anti-dsDNA antibodies [243]. Dietary factors have been shown to predispose to development of SLE in animals; cynomolgus macaques that were fed alfalfa sprouts developed an SLE-like illness [244]. The key constituent of alfalfa to induce this effect appeared to be the amino acid L-canavanine [245].

Viral infections have been implicated in the pathogenesis of several autoimmune diseases, including diabetes mellitus and SLE, and an association was reported between Epstein-Barr virus (EBV) infection and SLE in children and young adults [246]. There is some evidence for the involvement of a transmissible agent in

canine lupus; cell-free spleen extracts from dogs with lupus resulted in the generation of ANAs when given to newborn puppies [247]. This led to the suggestion that human SLE might be a zoonosis transmitted from dog to man. However, despite suggestions of immunological abnormalities in dogs belonging to humans with lupus [248,249], this was not confirmed in other large, well-controlled studies [250-252].

Despite evidence that environmental factors influence susceptibility to SLE in humans, no single environmental agent responsible for SLE has consistently been identified, with the exception of the rare cases of drug-induced disease.

Pathogenesis of SLE – Mechanisms responsible for the autoimmune response

Early studies demonstrated that a tendency to produce autoantibodies and develop glomerulonephritis could be transferred from NZB mice to normal mice by bone marrow cells [253]. These experiments did not address the specific cell types involved and it is likely that both myeloid and lymphoid cells were transferred.

The importance of B lymphocytes in murine lupus was shown by abrogation of disease in MRL/lpr mice that were genetically manipulated to be deficient in B cells [254]. Evidence for the pathogenicity of autoantibodies in mice and humans, in particular anti-DNA antibodies, comes from several observations; tolerisation to DNA abrogates disease in NZB/W mice [255], injection into normal mice of anti-DNA antibodies from lupus-prone mice results in renal inflammation [256], and

anti-DNA antibodies can be eluted from human renal tissue and are concentrated compared to their titre in serum [257,258].

There is extremely strong evidence that the mature autoantibody response in SLE is driven by antigen, which is explored in more detail below. However, there is also evidence from mouse and human studies of polyclonal B cell activation which is likely to reflect abnormalities in the threshold for activation of lymphocytes and may play a role in disease susceptibility. The earliest immunological abnormality that can be identified in young NZB/W mice is polyclonal B cell activation [259]. The idea that polyclonal B cell defects may contribute to the pathogenesis of SLE is suggested by the fact that mutant mice with defects of B cell negative regulation develop a lupus-like disease. These include mice deficient in Lyn, CD22, SHP-1 and FcγRIIB. Environmental polyclonal activators such as pristane and endotoxin have also been shown in animal models to accelerate or augment lupus-like disease, although in the case of endotoxin which has wide ranging effects, it is difficult to attribute this to polyclonal activation alone.

Two important observations in humans suggest a role for T cells in the pathogenesis of SLE. Firstly, the association of disease with MHC class II [260] and secondly, the nature of the autoantibodies found in SLE which show features associated with T cell responses including isotype-switching and somatic mutation [261,262]. Several experimental approaches have subsequently demonstrated the T cell requirement more directly. Antibodies against CD4 abrogate disease in both MRL/lpr and

NZB/W mice, illustrating the role of T helper cells in both models [263]. Furthermore, MRL/lpr mice deficient in MHC class II have decreased susceptibility to disease compared to unmanipulated MRL/lpr mice [264], and manipulations to inhibit B and T cell interaction with CTLA4-Ig or anti-CD40 ligand reduce expression of disease in NZB/W mice [265-267]. These data show that the IgG anti-dsDNA autoantibodies in SLE bear the characteristics of an antigen-driven, T cell-dependent immune response, and that T helper cells are important in the provision of help required to generate such autoantibodies.

Native DNA is poorly immunogenic [268] and deliberate immunisation with DNA does not lead to SLE suggesting that DNA is only a target antigen and not the primary immunogen in SLE. The isolation of autoreactive T cells from SLE patients recognising cationic DNA-binding proteins, and the finding that nucleosome-primed T cells emerged spontaneously in lupus-prone mice before the serologic manifestations of autoimmunity, thus predicting the development of lupus nephritis well in advance, led to the hypothesis that DNA-protein complexes are the major immunogen in SLE [269-272]. In addition, Datta *et al* found that immunisation of pre-autoimmune lupus mice with pure mononucleosomes accelerated development of severe lupus nephritis [273]. This hypothesis was further strengthened by Burlingame *et al* who demonstrated, using serial serum samples from MRL/lpr and BXSB mice, that early autoantibodies recognised discontinuous epitopes on native chromatin and the (H2A-H2B)-DNA subnucleosome and that as the immune response progressed, native DNA and other chromatin constituents generally became

antigenic [274]. The same group compared the antigenicity of whole chromatin, DNA, denatured individual histones, and histone-histone and histone-DNA subnucleosome complexes and found that sera from patients with SLE reacted most frequently with whole chromatin, and that adsorption with chromatin removed the majority of reactivity with subnucleosome complexes and native DNA [275].

The source of autoantigens

As discussed above, there is much evidence that the autoimmune response in SLE is driven by antigen and that the initial immunogen consists of DNA-protein complexes. What is the source of these autoantigens which are ubiquitous and abundant intracellular components of all healthy tissues in the body?

Growth, development and repair all depend on the ability to remodel tissues. Cell death is a key feature of each of these processes and this normally occurs by the mechanism of apoptosis, or programmed cell death. Under normal circumstances, apoptotic cells are efficiently removed from tissues by uptake by phagocytosis by other cells by means of a variety of specific receptors [276].

A breakthrough in the recognition of the possible source of the autoantigens of SLE came with the observation by Casciola-Rosen *et al* that many of the most characteristic "intracellular" autoantigens of SLE were present in surface blebs of cells undergoing apoptosis [277]. This led to the hypothesis that it might be apoptotic cells and bodies that are the source of the autoantigens that drive the

autoimmune response in SLE. This hypothesis is further supported by the fact that several of the mouse models of SLE result from mutated genes encoding proteins which regulate development of apoptosis [238,239]. For example, the presence of the *lpr* gene, which encodes a mutated *fas* gene, dramatically enhances susceptibility to lupus in the MRL strain of mouse [238]. Furthermore, Elkon's group demonstrated that intravenous administration into normal mice of syngeneic apoptotic thymocytes induces autoantibody production [278], and defective *in vivo* clearance of injected apoptotic thymocytes was demonstrated in lupus-prone C1q-deficient mice [279]. Increased lymphocyte apoptosis was reported some time ago in humans with SLE [280]; more recently however, defective clearance of apoptotic cells has been suggested as an important event in the aetiopathogenesis of human SLE [281].

During apoptosis, various potential autoantigens in SLE become modified, which may result in enhancement of their immunogenicity [282]. In particular, apoptosis induced by the action of granzyme B was shown to modify many of the targets of systemic immune responses [283], and it is proposed that apoptosis might thus render cryptic epitopes immunodominant and lead to antigen presentation of epitopes to which the immune system has not achieved tolerance [284].

Immune complex-mediated inflammation in SLE

Anti-DNA and anti-nucleosome antibodies are thought to be a major cause of tissue damage in SLE and many studies have addressed how they may cause

glomerulonephritis. Anti-DNA antibodies have been eluted from kidneys of both humans [258] and mice [285] with lupus. However, anti-DNA antibodies are neither necessary nor sufficient for disease and humans, as well as certain strains of mice, may have high titres of anti-DNA antibodies without evidence of nephritis. In addition nephritis may be present in the absence of circulating anti-DNA antibodies. The mechanism by which autoantibodies bind to the glomeruli of patients with SLE is debated. The glomerular basement membrane is negatively charged and it has been suggested that positively charged antibodies with an alkaline pI may preferentially locate there [286]. However, other studies have not confirmed this [287].

Patients with SLE have a high incidence of DNA [258,288] and histones [289] present in the glomeruli compared to controls. The presence of increased nuclear material within lupus kidneys is consistent with several different proposed models of immune complex localisation in glomeruli, and there is evidence from rodent perfusion studies for each of them. They include complexes of antibody and DNA binding to histones planted in glomeruli [290], complexes of antibody and intact nucleosomes binding to glomeruli [291,292], and binding of free antibody to planted nuclear material such as DNA or nucleosomes [293].

There is evidence from mouse studies that antibodies located in the glomeruli are of pathogenetic significance. In one such study, NZB/W mice were made deficient for Fcγ receptors I and III and did not develop glomerulonephritis despite deposition of

immune complexes [294]. However, simultaneous studies have shown that complement activation by immune complexes in experimental models is not necessary for the development of glomerulonephritis [295].

Interaction of pentraxins with nuclear constituents

Despite the potential importance of exposure of the immune system to nucleosomes and chromatin, little is known about the mechanisms of clearance and processing of nucleosomes and chromatin. Recent evidence suggests that the pentraxin family of proteins may have an important *in vivo* role in the clearance of nucleosomes and chromatin.

The first indication that the pentraxins could interact with nuclear antigens came from a study by Gitlin *et al* in which they detected CRP localised to cell nuclei in synovial tissues from patients with rheumatoid arthritis [296]. The pattern of staining was not clearly defined, although the authors showed that the CRP was not produced *in situ*. The first direct evidence that pentraxins could interact with nuclear antigens came from the work of Robey *et al* [297]. They demonstrated that rabbit CRP bound in a spotty pattern to the nuclei of rabbit skin and lung fibroblasts treated with bradykinin, phospholipase A_2 or lysolecithin to permeabilise them. They attributed this binding to chromatin and showed that binding to isolated chromatin from rabbit nuclei was inhibited by excess CRP or by phosphocholine, therefore implying that CRP may have a role in handling chromatin.

In 1987, Pepys' group demonstrated that SAP is the major calcium-dependent specific DNA binding protein in the serum, by demonstrating that human SAP was the only protein in normal or acute phase (containing high levels of CRP) serum that underwent binding under physiological conditions of pH and ionic strength to either single-stranded or double-stranded DNA immobilised on cellulose, on agarose beads or covalently coupled to Sepharose [60]. No specific binding of CRP from acute phase serum was detected and when the same experiments were repeated using sera from various other animals (mouse, rat, guinea pig, cow and plaice), all demonstrated specific calcium-dependent binding of SAP to DNA. In contrast, CRP from the rat, rabbit, plaice and horse-shoe crab, as well as hamster female protein, a homologue of CRP [298], did not bind to DNA, thus resembling the behaviour of human CRP. Isolated purified human SAP also showed major calcium-dependent binding to native long chromatin, H1-stripped chromatin and native DNA at physiological ionic strength, and when immobilised on Sepharose, to core particles in solution, whereas CRP only bound in trace amounts. Binding of SAP to extracellular chromatin in the skin lesions of two patients with SLE [299] and to dermal keratin bodies, that is apoptotic cells, in normal skin [300] has been demonstrated.

There has been considerable controversy over the ability of CRP to bind to chromatin. Such binding was demonstrated by Du Clos' group using chromatin from calf thymus rather than from chicken erythrocytes. Avian chromatin contains histone H5 instead of histone H1, and the amino acid sequences are very different.

Du Clos' group have shown that the binding of CRP to chromatin is entirely dependent on the presence of histone H1 [301] and CRP therefore cannot bind to avian chromatin. These binding studies [301-304], however, were carried out using isolated, purified nuclear constituents immobilised under conditions that could lead to denaturation [77] or performed in unphysiological buffers [297], and although the results may be interesting with regard to the potential for interaction of different molecules, they may not reflect the interactions that occur *in vivo.* Du Clos' group has also examined CRP binding to chromatin subunits and shown that CRP can bind to the H2A-H2B dimer and the $(H3\text{-}H4)_2$ tetramer and to H1, all interactions being inhibited by DNA. CRP preferentially bound to histones H1 and H2A and a short 8 amino acid sequence was defined on histone H2A which bound CRP in a calcium-dependent manner. Hamster female protein was shown to bind to H1, H2A, chromatin and DNA, all interactions being inhibited by phosphocholine [305].

Solubilisation of chromatin by pentraxins

The first report of pentraxins solubilising chromatin was from Robey *et al* who showed that CRP-chromatin complexes could consume complement at physiological concentrations of CRP and that this reaction was strictly complement dependent through the classical complement pathway [306]. However all these studies were conducted at sub-physiological ionic strength and are not likely to reflect meaningful interactions *in vivo.* Indeed Pepys and colleagues showed that SAP rather than CRP could solubilise native long chromatin under physiological conditions [61]. Unlike the earlier reports from Robey, there was no requirement for complement and no

cleavage of chromatin during these experiments. The binding of SAP to chromatin resulted in the displacement of H1-type histones (H5) essential for condensation and higher order folding of chromatin. This binding is remarkable as displacement of H1 and H5 by salt alone requires 0.5M NaCl. SAP also bound to nucleosome core particles forming soluble complexes with an apparent stoichiometry of 1:2, a result that is compatible with the attachment of SAP at the nucleosome dyad, the site of H1 in intact chromatin. CRP did not bind significantly to chromatin or core particles at physiological ionic strength. SAP may thus undergo a specific, avid interaction with chromatin and nucleosome core particles *in vivo*, and promote their solubilisation and normal handling when released from dying cells.

Binding of pentraxins to extractable nuclear antigens

The spotty fluorescence reported by Robey was recognised as being similar to that observed by the binding of autoantibodies to extractable nuclear antigens and in 1989 Du Clos demonstrated that CRP bound to nuclei of tissue culture cells that had been fixed and permeabilised with an identical pattern to that seen with anti-RNP binding, suggesting recognition of the same nuclear particles [307]. Using immunoprecipitation, CRP was shown to precipitate the U1 RNA species and the U4-U6 species characteristic of autoantibodies to the small nuclear ribonuclear protein complex, Sm/RNP. By blotting analysis, CRP reacted with the D protein of Sm and the 70 kDa protein of RNP.

These studies were confirmed by Pepys *et al* in 1994 using confocal microscopy, monoclonal antibodies and whole acute phase serum to show that CRP bound exclusively to small nuclear ribonucleoprotein particles and SAP bound only to chromatin and, for the first time, to nucleoli [62]. Together with the evidence that SAP is deposited *in vivo* in extracellular accumulations of chromatin from dead cells [299], this was further evidence of a possible functional role of pentraxins in handling nuclear material.

Binding of pentraxins to Fc-gamma receptors

Salmon *et al* noted in 1996 that the Fc-gamma receptor IIA (FcγRIIA) gene has 2 codominantly expressed alleles, R131 and H131, which differ in their ability to ligate human IgG2. They hypothesised that the FcγRIIA genes are important disease susceptibility factors for SLE, and subsequently demonstrated a reduced frequency of H131 homozygotes among African-American lupus patients compared to African-American non-SLE controls [227], a finding which has been repeated in other studies [308].

The binding of CRP to intact human cells via a specific receptor has been the subject of considerable controversy. As binding was found on predominantly FcγR expressing cells, FcγR was suggested to be the CRP receptor [92,309,310]. However, several authors presented data arguing against this hypothesis [193,311-313]. More recently, low and high affinity binding of CRP to human FcγRI and FcγRII has been reported by Du Clos' laboratory [92,93]. They also reported

decreased CRP binding to cells from FcγRIIA H131 homozygotes, and postulated that the reciprocal relationship between CRP and IgG binding avidities on leukocytes might explain the contribution of FcγRIIA alleles to SLE susceptibility [314]. In all these studies however, binding to FcγR was demonstrated by using human CRP preparations that had not been rigorously shown to be absolutely pure and also by IgG antibody-dependent detection methods. More recently, Hundt *et al* demonstrated that highly purified natural CRP and recombinant human CRP did not bind to white blood cells, but that even trace contamination of the CRP with IgG reproducibly gave the apparent binding of CRP to leukocytes observed by Du Clos [95]. These results are consistent with earlier work from our laboratory in which we were unable to detect any significant binding or association of purified CRP with the surface of intact peripheral blood leukocytes (M.B. Pepys, unpublished observations).

Defective CRP response in SLE

In a number of autoimmune and inflammatory diseases, such as rheumatoid arthritis, Crohn's disease and rheumatic fever, CRP concentration is the single most sensitive objective criterion of activity of disease [151,152,315,316]. When clinical symptoms and laboratory indices of disease activity are reduced, either spontaneously or following therapy, CRP levels tend to fall. CRP concentration rises and falls rapidly, over 24 to 48 hours, in response to acute changes in disease activity, covers a wide incremental range, and is not affected by the action of any of

the commonly used cytotoxic or anti-inflammatory drugs per se, unless these drugs reduce the activity or extent of tissue damage and inflammation [317].

In marked contrast to most autoimmune disorders, disease activity in SLE with severe symptoms and continuing tissue damage is associated with CRP levels which are raised only modestly, despite other tests for active inflammation being positive [110]. Honig *et al* found that definitive positive tests for CRP were rare in patients with SLE alone, regardless of its activity, but that if patients had evidence of an intercurrent microbial infection then the CRP test became strongly positive [318]. Subsequent studies have confirmed that although patients with SLE have significantly higher serum concentrations of CRP when their disease is active than when it is inactive, the maximum concentrations obtained are relatively modest, even in patients with extremely severe disease [156,160]. In contrast, patients with unequivocal evidence of intercurrent microbial infection, whether bacterial, viral or fungal, appear to mount an "appropriate" response of CRP to their infection, and CRP concentration is now frequently applied clinically to differentiate between inflammation associated with lupus activity itself and intercurrent infection in patients with SLE.

The mechanism for the poor CRP response in SLE is not known. However, *in vivo* turnover studies with ^{125}I-labelled CRP did not provide evidence of increased catabolism or consumption of CRP in SLE [159] and there is therefore, by definition, a reduced rate of synthesis. There may be failure of the disease process

in the tissues to generate the particular cytokine mediators which signal hepatocytes to synthesise and secrete CRP. However, an intriguing possibility is that the capacity to respond to particular forms of inflammation, tissue injury or other stimuli by production of CRP is genetically determined. There is evidence for such genetic control in inbred mice in which resting and acute phase levels of CRP [173] and SAP [116] are significantly different in different strains. In particular, the acute phase plasma protein response of SAP to autologous tissue damage in the NZB x W mouse strain which spontaneously develops a lupus-like phenotype was deficient when compared to the MRL *lpr/lpr* mouse which develops a phenotype resembling rheumatoid arthritis [319]. If this were true in man, it would raise the possibility that CRP-responder status could contribute to hereditary predisposition to SLE.

Biological significance of pentraxin interaction with nuclear antigens

Few studies have investigated the role of pentraxin binding to nuclear antigens *in vivo.* The effect of CRP on the course of autoimmune disease in the (NZB x NZW)F_1 female hybrid mouse was examined by Du Clos' group [320]. Mice were treated with intraperitoneal injections of chromatin immobilised on a solid phase matrix in the presence or absence of CRP. Injection of chromatin in the absence of CRP decreased the survival of 26-week old mice, from a median survival age of 37 weeks for controls to 29 weeks. CRP prevented this accelerated phenotype and mice injected with CRP-chromatin had a median survival age of 40 weeks. Mice treated with CRP alone did not survive longer than controls and

pretreatment with CRP did not affect the clearance of nucleosome core particles in normal BALB/c mice.

The effect of the acute phase response on clearance of H1-stripped chromatin and nucleosome core particles was examined in different mouse strains, chosen due to their different basal concentrations of SAP [321]. Core particle clearance was found to be slow whereas chromatin clearance was rapid and varied in the different mouse strains. Induction of the acute phase response in C57BL/10 mice led to a decrease in chromatin clearance, as did co-injection with human SAP or human CRP. The acute phase response also caused a higher percentage of chromatin to localise to the liver compared to the spleen and less to localise to the kidney, suggesting a protective role of the acute phase response against renal deposition of chromatin. However, small numbers of animals were used in this study and the conclusions reached were not convincingly demonstrated by the actual observations.

The spontaneous phenotype of mice with targeted deletion of the SAP gene was first reported from our laboratory [232]. A cohort of (129/Sv x C57BL/6)F_2 SAP deficient mice was noted to spontaneously develop antinuclear autoimmunity, including anti-chromatin, anti-histone and anti-DNA antibodies, with 41% of deficient female mice developing immune complex glomerulonephritis. The clearance of administered chromatin was also reported to be faster in SAP deficient animals compared to their wild-type counterparts. However, these studies were

performed in a heterogeneous group of mice and whether SAP deficiency in pure line mice is sufficient to cause autoimmunity has not been tested.

SCOPE AND HYPOTHESIS OF THIS THESIS

The pentraxin proteins are highly conserved through evolution suggesting an important physiological function *in vivo.* SAP is the single normal circulating protein that shows specific calcium-dependent binding to DNA and chromatin [60,299,300] and CRP binds specifically to small nuclear ribonucleoproteins [62,307]. Furthermore, a cohort of [C57BL/6 x 129/Sv]F_2 mice with targeted deletion of the SAP gene were recently discovered to develop spontaneous antinuclear autoimmunity and immune complex glomerulonephritis, a phenotype resembling human lupus [232]. Accompanied by the deficient CRP response to autologous tissue damage in patients with SLE [156,157], these results suggested that an important function of both SAP and CRP may be to facilitate the safe and non-immunogenic disposal of autologous ligands.

In this thesis the hypothesis that one of the physiological functions of SAP and CRP is to protect against autoimmunity is tested and attempts are made to elucidate the mechanisms involved. In particular, we determine for the first time the detailed spontaneous phenotype of pure-line C57BL/6 and 129/sv SAP deficient mice, testing the hypothesis that SAP deficiency in mice causes a lupus-like phenotype.
As highlighted earlier in the chapter, several lines of evidence suggest a role for apoptotic cells in the induction of autoimmunity [238,239,277-279]. Pentraxins

specifically bind nuclear autoantigens and in this thesis we confirm the binding of pentraxins to apoptotic cells *in vitro*.

One possible mechanism by which pentraxins may prevent systemic autoimmunity *in vivo* is by facilitating the non-immunogenic clearance of apoptotic cells by macrophages [276,322]. In order to test this hypothesis we compared the following: the immune response to administration of syngeneic apoptotic thymocytes between wild-type and SAP deficient mice *in vivo*; the phagocytosis of apoptotic thymocytes between SAP deficient and wild-type C57BL/6 pure-line mice *in vivo*; the phagocytosis of primary human apoptotic lymphocytes by macrophages *in vitro,* in the presence and absence of SAP.

Another possible mechanism by which pentraxins may prevent systemic autoimmunity is by enhancement of the non-immunogenic handling and disposal of nucleosome core particles that are known to be released into the circulation from cells dying by apoptosis [323,324]. In order to test this hypothesis we compared the following: the *in vivo* plasma clearance kinetics of administered long-chromatin and nucleosome core particles in SAP-/- and wild-type C57BL/6 mice; the sites of catabolism of nucleosome core particles in SAP-/- and wild-type mice.

Chapter 2 - Materials and Methods

MATERIALS

General Reagents

All reagents were obtained from BDH (Leicestershire, UK), Sigma-Aldrich (Poole, Dorset, UK), or Pharmacia Biotech (St Albans, UK) except for:

Agar	Difco Laboratories Ltd, West Molesey, UK
Indubiose A37 agarose	IBF/Uniscience Gennevilliers, France
Tween 20	Biorad Labs, Herts, UK
Cellulose DE52	Whatman International Ltd, Maidstone, UK

Enzymes

N-bromosuccinimide	British Drug Houses, Poole, UK
Staphylococcal nuclease S7	F. Hoffmann-La Roche Ltd, Basel, Switzerland

Kit Reagents

Nucleon DNA extraction kit	Life Sciences, UK
QIAamp^{R} DNA extraction kit	Qiagen Ltd, Crawley, UK
Annexin V apoptosis detection kit	Immunotech, Marseilles, France
FragEl^{TM} apoptosis detection kit	Oncogene, Boston, USA
Phycolink^{R} phycoerythrin (RPE)	ProZyme Inc, San Leandro, USA

Radiolabelled Isotopes and Autoradiography Reagents

The radiolabelled (^{125}I)sheep anti-mouse whole immunoglobulin and Na^{125}I isotope were supplied by Amersham Biosciences, Little Chalfont, UK.

Nuclear Emulsion solution	38 ml K5 nuclear emulsion (Ilford Ltd, Knutsford, UK) in 12 ml dH_2O
Rapid Fixer	Ilford Ltd, Knutsford, UK
LX24 Developer	Kodak, Hemel Hempstead, UK
Hyperfilm™	Amersham Biosciences, Little Chalfont, UK
Hypercassette™	Amersham Biosciences, Little Chalfont, UK

Cell Culture Media and Cell Lines

All cell culture reagents were obtained from Invitrogen Ltd, Paisley, UK except for:

Jurkhat cell line	From Dr M Botto, Hammersmith Hospital
Hep-2 cell line	From Dr M Botto, Hammersmith Hospital
Fetal Calf serum (FCS)	BioWhittaker Ltd, Verviers, Belgium
DMEM	Dulbecco's Modified Eagles Medium, BioWhittaker Ltd, Verviers, Belgium
Serum replacement	ICN Biomedicals, Aurora, USA
Trypsin	0.25% in HBSS, ICN Biomedicals, Aurora, USA
Lymphoprep™	Nycomed Pharma, Oslo, Norway
Cell Culture Flasks	Corning Costar Corp., Cambridge, USA

Common Buffers

Barbitone-EDTA (BE)	0.07 M Barbitone (0.0603 M Na Barbitone, 0.0097 M Barbitone), 0.01 M di-tetra EDTA, 0.1% Na azide (pH 8.6)
EDTA	Equimolar mixture, pH 7.5, of disodium and tetrasodium ethylene diamine tetra-acetate
PBS	Phosphate buffered saline, 0.2 g potassium phosphate monobasic, 0.2 g KCl, 8 g NaCl and 1.15 g sodium phosphate dibasic dissolved in 1 litre of dH_2O (supplied as a powder mix by Sigma-Aldrich)
PEBT	PBS containing 10 mM EDTA, 1% BSA, 0.2% Tween 20 and 0.1% sodium azide, pH 7.4
SDS-PAGE reducing buffer	0.01 M Tris, pH 8, 1 mM di-tetra EDTA, 2.5% v/w SDS, 0.025% v/w Bromophenol Blue and 0.7 M mercaptoethanol, 1% v/w glycerol
Sodium carbonate buffer	0.5 M mixture of disodium salt, pH, and monosodium salt, pH achieving final pH 9.5
TBE	Tris borate, 0.09 M Tris-borate, 0.02 M EDTA
TC	Tris calcium, 0.01 M Tris, 0.138 M NaCl, 0.002 M $CaCl_2$, pH 8.0
TCB	TC containing 1-4% w/v BSA (Sigma-Aldrich)
TE	Tris EDTA, 0.01 M Tris, 0.138 M NaCl, 0.01 M EDTA, pH 8.0

TEB	TE containing 1% w/v BSA (Sigma-Aldrich)
TN	Tris NaCl, 0.01 M Tris, 0.138 M NaCl, pH 7.4
Tris-HCl	0.01 M Tris, pH 8.0

Antibodies and Antigens

All antibodies, antigens and microtitre plates were purchased from Sigma-Aldrich (Poole, Dorset) except for:

Lyophilised calf thymus histones	Calbiochem Novabiochem, La Jolla, USA
Avian long chromatin	Prepared by Dr WL Hutchinson, CAAPP, Dept. of Medicine, Royal Free and University College Medical School, London [325,326]
MRL/Mp-*lpr/lpr* serum	Dr M Botto, Hammersmith Hospital, London
FITC-goat anti-mouse C3	Cappell-Worthington, Durham, NC
Crithidia luciliae slides	The Binding Site, Birmingham, UK
Hep-2 slides	The Binding Site, Birmingham, UK
Nunc plates	Nunc, Roskilde, Denmark
Sheep anti-human SAP	Monospecific antiserum raised by immunisation with the respective pure protein [66]
Sheep anti-mouse SAP	Monospecific antiserum raised by immunisation with the respective pure protein

Goat anti-human CRP	Monospecific antiserum raised by immunisation with the respective pure protein
Rabbit anti-mouse albumin	Biogenesis Ltd, Poole, UK
Mouse anti-human Fas	Immunotech, Marseilles, France

Histological Compounds

10% buffered formalin	Histopathology Dept., Royal Free Hospital
10% Giemsa stain	Haematology Dept., Royal Free Hospital
Haematoxylin and eosin stain	Histopathology Dept., Royal Free Hospital
Periodic acid Schiff's (PAS) stain	Histopathology Dept., Hammersmith Hospital
OCT compound	Sakura Finitek, Torrance, Ca
DPX mountant	Raymond Lamb, Eastbourne, UK

Histological Buffers

Bouin's solution	75 ml saturated 1.2% picric acid, 25 ml formaldehyde 40% w/v, 5 ml acetic acid, added before use
Electron microscopy fixative	1 ml 25% gluteraldehyde, added before use to 11.5 ml PBS

Animals

Pure line SAP-deficient mice were obtained by back crossing the original SAP knockouts [208] through at least 6 generations onto the background strain in a

specific pathogen free (SPF) environment. Other strains of inbred mice (C57/BL6 and 129/sv) were purchased from Harlan Olac, Bicester, Oxon, UK. All experiments involving the use of animals were conducted in accordance with local ethical guidelines and Home Office regulations.

METHODS

Gel Electrophoresis

Agarose gel electrophoresis

Electrophoresis grade agarose was dissolved in 1xTBE to a final concentration of 0.8-2% depending on the size range of the DNA fragments to be resolved (Sambrook et al 1989). The agarose solutions were cooled to 50°C and ethidium bromide (EtBr) was added to a final concentration of 0.5 μg/ml before the gels were set in horizontal gel trays. Sample loading buffer was added to all samples prior to loading. A "1 Kb ladder" was added to marker lanes for size estimation. Electrophoresis was carried out in 1 x TBE under a constant voltage of between 2.5 and 5.0 V/cm.

Gels were visualised using a short-range UV transilluminator (312 nm) (Ultraviolet Products Ltd, Cambridge, UK), electronically captured using the Visionworks Image Acquisition Software (Ultraviolet Products Ltd, Cambridge, UK) and recorded on thermosensitive paper using a digital graphic printer (Sony Corporation, Tokyo, Japan).

Electroimmunoassay

Electroimmunoassay was performed by the standard method [327], using 1% indubiose agarose containing the relevant monospecific antiserum at a concentration of 1-2%. Serum samples (2 µl) were placed in 2 mm diameter wells on a 1 mm thick gel and a constant voltage of 200 V was applied for 8 h across the gel, in an electrophoresis tank with cooled plates containing barbitone-EDTA and using double thickness 3MM^{TM} chromatography paper as a contact wick between buffer and gel. The gel was washed overnight at 37°C with 5% NaCl, 0.02% NaN_3 to remove unprecipitated protein, and then pressed and dried. The gel was stained with 0.2% Coomassie blue and destained (45% ethanol, 10% glacial acetic acid) until the background of the gel was clear. Rocket shaped curves occur with the precipitation of the antigen-antibody complex at a critical antigen-antibody ratio (equivalence). The heights of the "rockets" are proportional to the concentration of antigen in each sample and are compared to known concentrations of the antigen in standards of serum, calibrated against the pure antigen.

Denaturing polyacrylamide gel electrophoresis

Samples for protein analysis were diluted in SDS-PAGE reducing sample buffer and heated at 95°C for 5 min. The electrophoresis unit was cooled to 15°C by connecting to a thermostatic circulator. The electrophoresis gel (*ExcelGel,* Pharmacia Biotech, St Albans, UK) was positioned on the cooling plate using silicone fluid (BDH), and the SDS buffer strips were applied to the appropriate side of the gel. Samples estimated to contain 200-500 ng of each protein and appropriate low molecular

weight (LMW) calibration standards (Pharmacia Biotech, UK) were applied to the gel through application pieces, and run at 600 V with a current of 50 mA and power of 30 W for 1-2 h. The gels were subsequently placed directly into Coomassie Staining solution (0.1% Coomassie Blue in 30% methanol, and 10% acetic acid) at 56°C for 30 min and washed once with distilled water before destaining. Silver staining was performed where necessary according to the method of Heukeshoven and Dernick [328].

Phenotypic Analysis of SAP-deficient Mice

Serological analysis

Blood was collected by tail bleeding (200 µl per bleed) or at bleed out into 1.5 ml vials (Eppendorf, Hamburg, Germany) and allowed to clot for 6 h at room temperature. Serum was separated by centrifugation at 14000 rpm in a microfuge (Sigma-Aldrich, Poole, UK) for 5 min, and stored in aliquots at -70°C prior to analysis.

Immunoradiometric assays (IRMAs) were developed and optimised with the help of Dr WL Hutchinson [329], Centre for Amyloidosis and Acute Phase Proteins, Royal Free and University College Medical School, London. All such assays used immobilised pure antigen as an initial capture phase for specific murine antibodies. Detection was achieved using radiolabelled sheep anti-mouse IgG which was estimated by counting individual reaction wells in a gamma counter (Cobra^{TM} II, Packard, Pangbourne, UK).

The dilutions chosen for IRMAs were chosen to accommodate the volume of serum available and produce values throughout the range of detection. For each assay the intra-assay coefficient of variation was assessed by comparison of 2 samples measured 6 times per assay and the inter-assay coefficient of variation was assessed on the same samples measured on each occasion.

All samples were assayed in triplicate and results were expressed relative to those produced by a standard of pooled, high titre MRL/Mp-*lpr/lpr* serum which was assigned an arbitrary value of 100 units. Samples were considered positive when > 3 SD above the lower limit of detection.

Anti-nuclear antibodies (ANA) – by indirect immunofluorescence

HEp-2 cells were grown to confluence at 37°C in RPMI medium containing 10% heat inactivated fetal calf serum, penicillin G sodium 250 u/ml, streptomycin sulphate 200 μg/ml, amphotericin B 2 μg/ml and glutamate. Once confluent, cells were released from the cell culture flask by incubation at 37°C with 0.25% trypsin for 2-4 min, and gentle tapping. The cell suspension was diluted 1:5 with RPMI medium, centrifuged (Sorvall RT6000B) at 350 g for 5 min, the supernatant was discarded and cells resuspended in fresh RPMI medium. One ml of cell suspension per slide was then pipetted onto sterile multi-well slides in individual petri dishes and cultured at 37°C for a further 24-48 h. Once confluence had been attained, slides were removed, washed twice in sterile PBS, fixed with acetone for 5 min and stored at -70°C until use.

Samples were diluted 1/80 with sterile PBS. MRL/Mp-*lpr/lpr* serum was used as a positive control and pooled serum from normal mice as a negative control. Multi well slides, bearing HEp-2 cells, were preincubated with 15 µl samples in a wet box and left for 1 h at room temperature. The slides were washed twice in PBS for 5 min and incubated in the dark for 30 min with 400 µl of FITC-conjugated goat IgG anti-mouse Fc secondary antibody, diluted 1/120 [330,331]. The slides were washed in PBS, a coverslip applied with Permafluor (Immunon, UK) and the samples viewed in a fluorescence microscope (Olympus BH/2). Positive samples were then serially diluted and titrated to end-point (i.e. the last dilution at which a positive result was demonstrated). Results were expressed as titration units. Each assay was standardised using positive pooled MRL/Mp-*lpr/lpr* serum which was serially diluted from 1/80 to 1/2560. Values of 1/80 or above were considered positive.

Anti-dsDNA antibodies - by indirect immunofluorescence *(Crithidia luciliae)*

Samples were diluted 1/20 with sterile PBS. Pooled MRL/Mp-*lpr/lpr* serum was used as a positive control and pooled serum from normal mice as a negative control on every slide. The assay was standardised using a further sample of positive pooled serum and serially diluting from 1/20 to 1/160. The sample was positive at 1/160. Sample dilutions were placed into each well of commercially prepared slides bearing *Crithidia luciliae* and incubated in a humid chamber for 1 h. The slides were washed twice with PBS and 40 µl of FITC-conjugate at a dilution of 1/50 was added to each well (goat anti-mouse polyvalent immunoglobulins – IgG, IgA, IgM) and incubated again for 30 min in the dark at room temperature. The slides were washed

with PBS, a coverslip mounted with Permafluor and the slides visualised in a fluorescence microscope. Positive samples were titrated to end-point. Values of 1/20 or above were considered positive [332].

Anti-histone antibodies – by immunoradiometric assay (IRMA)

Microtitre plates with N-oxysuccinimide activated surfaces (Corning Costar, Bucks, UK) were coated with calf thymus histones by incubation with 100 µl per well of the antigen at 100 µg/ml in PBS, pH 9.0, for 1 h at 21°C. After decanting, the wells were each washed twice with 200 µl of PBS, pH 7.4, containing 0.05% Tween 20, before blocking for 30 min with 100 µl per well of 20 g/l bovine serum albumin (BSA) in PBS, pH 7.4. After finally rinsing each well three times with 200 µl volumes of PBS, the plates were used for assays within 7 days. Stability of coated plates was confirmed by repeating assays 7 days apart.

Calibrators were constructed containing dilutions at 1/50, 1/100, 1/200, 1/500, 1/1000, 1/2000, 1/4000 and 1/8000 of pooled MRL/Mp-*lpr/lpr* serum in PEBT buffer. Serum samples for the assay were each diluted 1/200 in PEBT buffer (1/200 dilution of the standard corresponded to the top of the linear region of the sigmoid curve). The standards and diluted samples, together with PEBT buffer alone, were loaded in triplicate into the histone coated plates at 100 µl per well. After incubation at 37°C for 1 h, the plates were decanted and each well was washed three times with 200 µl of PEBT buffer before finally being blotted dry and counted individually in the γ-counter. The calibration curve was constructed using a four parameter logistic

curve fit program (figure 2.1a), and values for samples and controls were determined by interpolation.

The calibrators yielded a response proportional to the dilution used between 1/2000 and 1/200. Readings reached a plateau for calibrators at dilutions lower than 1/200, and higher than 1/2000. Intra-assay coefficients of variation were between 1.77% and 7.6% and inter-assay coefficients of variation ranged from 1% to 8.4%.

Anti-chromatin antibodies – by immunoradiometric assay (IRMA)

Microtitre plates with N-oxysuccinimide activated surfaces (Corning Costar, Bucks, UK) were coated with avian long chromatin by incubation with 100 µl per well of the antigen at 100 µg/ml in PBS, pH 9.0, for 1 h at 21°C. After decanting, the wells were each washed twice with 200 µl of PBS, pH 7.4, containing 0.05% Tween 20, before blocking for 30 min with 100 µl per well of 20 g/l bovine serum albumin (BSA) in PBS, pH 7.4. After finally rinsing each well three times with 200 µl volumes of PBS, the plates were used for assays within 7 days. Stability of coated plates was confirmed by repeating assays 7 days apart.

Calibrators were constructed containing dilutions at 1/200, 1/400, 1/800, 1/1000, 1/2000 and 1/4000, 1/8000 and 1/16000 of pooled MRL/Mp-*lpr/lpr* serum in PEBT buffer. Serum samples for the assay were each diluted 1/1000 in PEBT buffer (1/1000 dilution of the standard corresponded to the top of the linear region of the sigmoid curve). The standards and diluted samples, together with PEBT buffer

alone, were loaded in triplicate into the chromatin coated plates at 100 µl per well. After incubation at 37°C for 1 h, the plates were decanted and each well was washed three times with 200 µl of PEBT buffer before finally being blotted dry and counted individually in the γ-counter. The calibration curve was constructed using a four parameter logistic curve fit program (figure 2.1b), and values for samples and controls were determined by interpolation.

The calibrators yielded a response proportional to the dilution used between 1/1000 and 1/4000. Readings reached a plateau for calibrators at dilutions lower than 1/1000, and higher than 1/4000. Intra-assay coefficients of variation were between 1.2% and 5.7% and inter-assay coefficients of variation ranged from 3.3% to 11.7%.

Anti-ssDNA antibodies – by immunoradiometric assay (IRMA)

Calf thymus single-stranded DNA (ssDNA) (Sigma, Poole, UK) was dissolved at 100 µg/ml in PBS, pH 9.0 by heating to 70°C for 30 min. Microtitre plates with N-oxysuccinimide activated surfaces (Corning Costar, Bucks, UK) were coated by incubation with 100 µl per well of the antigen for 1 h at 21°C. After decanting, the wells were washed, blocked and washed further, before use within 7 days as before. Stability of coated plates was confirmed by repeating assays 7 days apart.

Calibrators were constructed containing dilutions at 1/200, 1/400, 1/800, 1/1000, 1/2000 and 1/4000, 1/8000 and 1/16000 of pooled MRL/Mp-*lpr/lpr* serum in PEBT buffer. Serum samples for the assay were each diluted 1/1000 in PEBT buffer

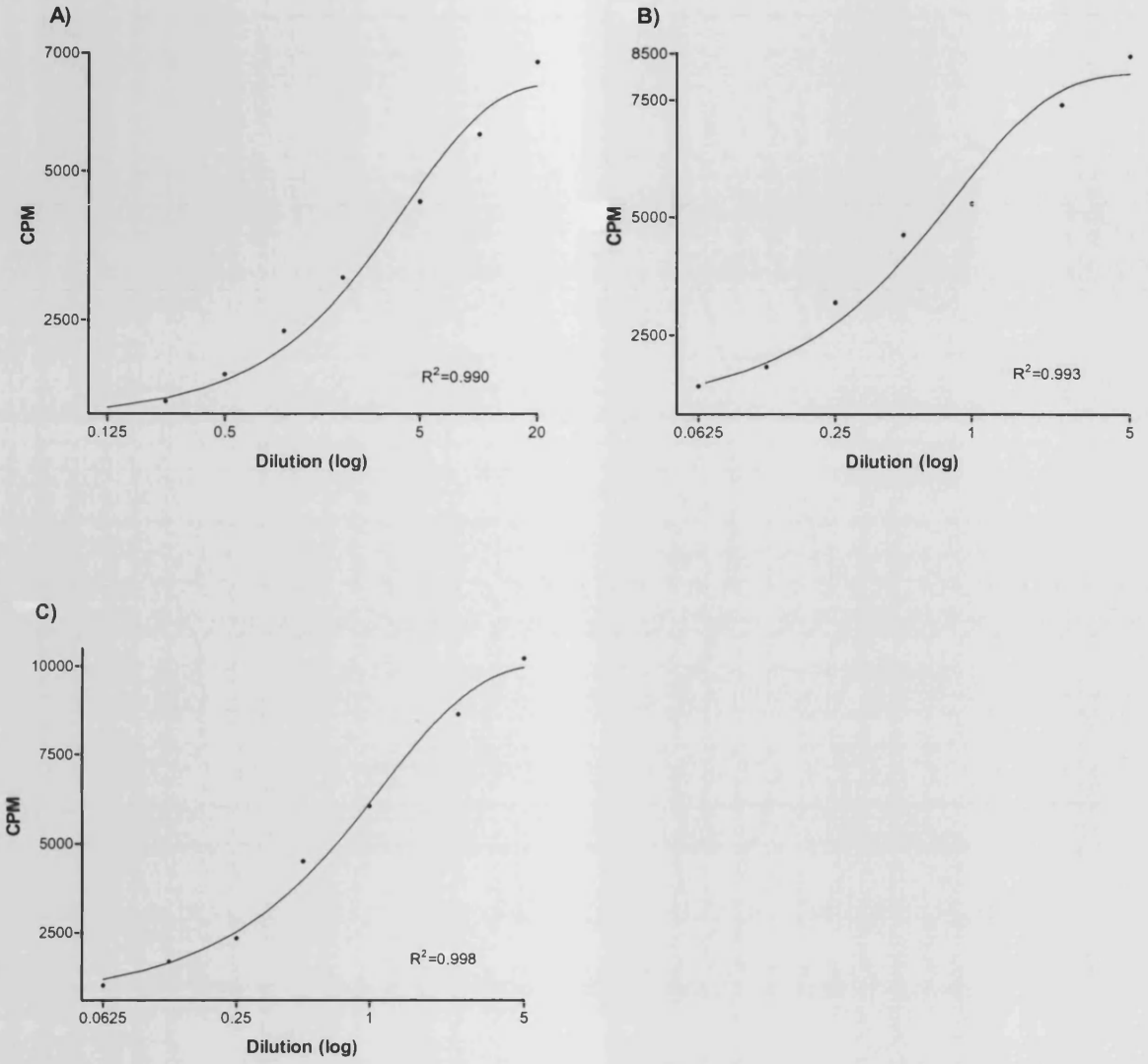


Figure 2.1 Calibration curves for immunoradiometric assays (IRMAs) using serial dilutions of pooled MRL/Mp-lpr/lpr serum. A) Anti-histone antibody assay calibration curve B) Anti-chromatin antibody assay calibration curve C) Anti-ssDNA antibody assay calibration curve. Correlation coefficients (R^2) are shown for individual curves.

(1/1000 dilution of the standard corresponded to the top of the linear region of the sigmoid curve). The standards and diluted samples, together with PEBT buffer alone, were loaded in triplicate into the ssDNA coated plates at 100 µl per well. After incubation at 37°C for 1 h, the plates were decanted and each well was washed three times with 200 µl of PEBT buffer before finally being blotted dry and counted individually in the γ-counter. The calibration curve was constructed (figure 2.1c), and values for samples and controls were determined by interpolation, as before.

The calibrators yielded a response proportional to the dilution used between 1/1000 and 1/4000. Readings reached a plateau for calibrators at dilutions lower than 1/1000, and higher than 1/4000. Intra-assay coefficients of variation were between 0.1% and 2.5% and inter-assay coefficients of variation ranged from 0.5% to 5.8%.

Urinary analysis

Urine was collected from each C57BL/6 mouse over a 24 h period prior to exsanguination using a modified cage (Dr M Robson, Department of Rheumatology, Imperial College School of Medicine). The total volume of urine was measured and serum and urine creatinine concentrations were determined using a standard Olympus AU600 instrument. Creatinine clearance was calculated for each mouse using the standard formula GFR = [UCr x V]/PCr, where UCr and PCr are the urine and plasma creatinine concentrations respectively, and V is the volume of urine in 24 h. Creatinine clearance was expressed in ml per minute.

Urinary albumin concentration was determined by single radial immunodiffusion [333] using a 1.2% agarose gel containing rabbit anti-mouse albumin (Biogenesis) at a concentration of 1.5%. The assays were calibrated with standards of murine albumin in solution in mouse urine. Inter-assay variation was determined by comparison of the same two samples on each gel and was < 6%. Albuminuria was expressed in μg/ml with a lower cut off point of 50 μg/ml.

Histological analysis

Preparation of samples

Blocks of mouse liver, spleen, kidney, heart, lung, large intestine, small intestine, stomach salivary gland, aorta and skin were obtained and prepared in several ways (as required):

a. Fixed in 10% buffered formalin, prior to processing in paraffin, sectioning and staining with haematoxylin and eosin.
b. Snap frozen – from 0.9% saline on cork boards with OCT compound, by immersion in isopentane, cooled in liquid nitrogen. All tissues were stored at -70°C.
c. Fixed in Bouin's solution – blocks of mouse kidney were immediately immersed in Bouin's solution and left for 4 h and then stored in 70% ethanol, prior to processing in paraffin, sectioning and staining with haematoxylin and eosin or Periodic Acid Schiff (PAS).

d. Fixed in glutaraldeyde - blocks of mouse kidney were cut into 1 mm cubes and fixed in 2% glutaraldehyde in PBS at 4°C for 2-3 h, before storage in PBS. These were used for electron microscopy.

Analysis of samples

Light microscopy

Sections fixed with Bouin's solution or 10% buffered formalin were coded and read blind by Professor HT Cook, Department of Histopathology, Hammersmith Hospital. Kidney sections were scored for glomerulonephritis by grading glomerular hypercellularity in the following way: 0 = < 25%, I = 25-50%, II = 51-90% and III = > 90% abnormal glomeruli (Jevnikar 1992). Other tissues were evaluated for lymphocytic infiltration and scored according to the proportion of tissue infiltrated.

Splenic samples were coded and read blind by Dr RP Hasserjian, Department of Histopathology, Hammersmith Hospital, and were given a "spleen lymphocyte score" as follows: white pulp volume scored from 0 (none) to 3 (very abundant); white pulp coalescence scored from 0 (completely separated white pulp nodules) to 3 (totally coalescent white pulp); red pulp lymphocytes scored as 0 (very rare) to 3 (numerous). The spleen lymphocyte score was the sum of all parameters, ranging from 0-9, and ≥ 4 was considered abnormal.

Direct Immunofluorescence Staining for IgG and C3

The presence of IgG and C3 deposits in renal tissue from mice with varying degrees of glomerulonephritis, was sought by direct immunofluorescence on 5 μm thick cryostat sections of unfixed renal tissue (Bright Instruments Ltd, Huntingdon, UK). Sections were left to air dry for at least 1 h at room temperature, fixed in acetone and allowed to dry. Sections were then blocked with 20% normal goat serum in PBS for 30 min at room temperature, prior to incubation for 1 h at room temperature with 50 μl antibody conjugate used at 1/40 (FITC-polyclonal goat anti-mouse IgG, Fc specific) or 1/50 (FITC-polyclonal goat anti-mouse C3). Sections were washed twice in PBS for 3 min each, mounted in permafluor and viewed under fluorescent light. Specificity of staining was established by fluid-phase absorption of the fluorescent antibody conjugate with an equal volume of the respective antigen in slight, 1.2:1, antigen excess. The mixture was incubated at 37°C for 1 h and then rotated overnight at 4°C, centrifuged and the SN was removed and diluted to the same dilution as the unabsorbed labelled antibody for staining.

Serum from SAP-deficient and wild-type mice, that were strongly ANA positive and negative respectively, were incubated with snap-frozen cryostat sections of unfixed normal mouse tissues (liver, kidney, spleen) from C57BL/6 mice. After washing, these composite blocks were incubated with FITC-labelled sheep anti-mouse IgG and viewed under fluorescent light to detect the presence of tissue specific autoantibodies in the serum.

Immunisation with avian native long chromatin

Groups of age-matched male and female SAP deficient (SAP-/-) and wild-type 129/sv mice were injected with 100 μg chicken erythrocyte native long chromatin in 50 μl 10 mM Tris, pH 8.0, emulsified in an equal volume of complete Freund's adjuvant, intramuscularly into the right thigh on day 0. All mice were bled on day -1, +14, +28 and +41. They were boosted with the same dose of chromatin suspended in incomplete Freund's adjuvant on day +32 and bled out on day +56.

Plasma Clearance and Organ Localisation of Radiolabelled Chicken Erythrocyte Chromatin and Nucleosome Core Particles

Obtaining long chromatin and nucleosome core particles

Chicken erythrocyte long chromatin was prepared by Dr WL Hutchinson by limited digestion with exogenous staphylococcal nuclease according to the protocol of Dr S Searles (LMB, Cambridge), and stored at a concentration of 4.6 mg/ml in 10 mM Tris-HCl, pH 8.0 with glycerol to 50% v/v at -30°C. Aliquots were taken as necessary and chromatin was dialysed out of glycerol and into 10 mM Tris-HCl at 4°C using benzoylated dialysis tubing (Sigma-Aldrich, Poole, UK).

Nucleosomes were obtained from native long chromatin as follows; calcium chloride in Tris-HCl was added to the dialysed chromatin to a final concentration of 1 mM. After vortex mixing, the intact long chromatin was allowed to precipitate at room temperature for 30 min. EDTA was added to a final concentration of 2 mM, the

sample was microcentrifuged at 14000 rpm, and the supernatant (containing nucleosomes) was removed.

The DNA content of the supernatant was estimated by diluting 1:20 in 0.1 M NaOH and reading the optical density at A_{260} and A_{320} using the Beckmann DU650 spectrophotometer. In order to determine the size range of the DNA fragments in the supernatant, approximately 50 µg of DNA was extracted from an appropriate aliquot using the BACC2 DNA extraction kit (Life Sciences, UK). The DNA fragments were analysed by agarose gel electrophoresis alongside a "1 Kb ladder." The presence of H1-type histones in the nucleosome preparations was confirmed by SDS-PAGE.

Direct radioiodination of long chromatin and mononucleosomes

Long chromatin was directly radioiodinated as follows: following dialysis into 10 mM Tris, pH 8, 1 mg aliquots of chromatin were reacted with 5 µl $Na^{125}I$ (neutralised with 1 M Tris, pH 8) (Amersham International plc) and 5 µl N-bromosuccinimide (NBS) at 1 mg/ml for 20 sec before dilution to a final volume of 4.4 ml with 10 mM Tris, pH 8. Extensive dialysis was then carried out in Tris buffer in the presence of Amberlite IRA-420 anion exchange resin (100 ml in 5 litres buffer). Dialysis was undertaken for 96 h with 30 litres of buffer. The final 5 litre dialysis was carried out in the absence of resin. The proportion of radioactivity associated with protein in the final material was determined by precipitation with 30% trichloroacetic acid (TCA) and counting of the precipitate.

Nucleosomes were directly radioiodinated as follows; a Sephadex G-25 pre-packed 10 ml mini-column (PD10, Amersham Pharmacia Biotech, Uppsala, Sweden) was equilibrated in TN buffer, blocked with 20% BSA and re-equilibrated in TN buffer. Twelve µl of cold KI at 10 µg/ml in dH_2O was added to 200 µl (200 µg) of nucleosome solution. Three µl of $Na^{125}I$ (activity of 100 mCi/ml) (Amersham International plc) was added followed by 12 µl of N-bromosuccinimide (NBS). The reaction was allowed to proceed for 15 sec and was halted by addition of the solution to the equilibrated PD10 column. The PD10 column was eluted with TN and 0.5 ml aliquots were collected in separate fractions. The activity of each fraction was determined using a gamma counter and the two samples containing maximum activity were pooled. The final material was tested for TCA precipitability.

Synthesis of tyramine-cellobiose

This was performed according to the method of Pittman *et al* [334] and involved reductive amination of cellobiose by tyramine, using sodium cyanoborohydride as the reducing agent. Tyramine (100 mmol) in 1 litre dH_2O (0.1 M) was dissolved by stirring in 20 ml acetic acid. Sodium cyanoborohydride (100 mmol) and cellobiose (40 mmol) were added and the pH was adjusted to 5.4 with glacial acetic acid. The mixture was refluxed with stirring for 96 h with readjustment of the pH to 5.4 at 24 hourly intervals. The solution was cooled, adjusted to pH 3.5 and refluxed for a further 24 h to destroy any remaining sodium cyanoborohydride. The volume of the solution was reduced to approximately 100 ml by rotary evaporation. Following

centrifugation to remove any particulate matter, the concentrate was applied to a 120 ml column of AG 50W-X8 cation exchange resin (Biorad, Herts, UK), equilibrated with H_2O. The column was eluted with a minimum of 2.5 litres of water to remove unreacted cellobiose, and then with a linear gradient of H_2O and 1 M NH_4OH which elutes the tyramine-cellobiose ahead of unreacted tyramine. The elution pattern was monitored by thin layer chromatography on silica gel developed in butanol: acetic acid: H_2O (7:1:2). Tyramine-cellobiose was identified by staining with a mixture containing equal volumes of 2 mg/ml naphthoresorcinal in ethanol and 20% H_2SO_4. Fractions containing tyramine-cellobiose were pooled, rotary evaporated to about 60 ml and lyophilised to yield a powder and stored in a dessicator.

Radioiodination of the tyramine cellobiose ligand

To a 300 µl microreaction glass vessel, 400 ng of 1,3,4,6-tetrachloro-3α,6α-dipthenylglycouril (Iodogen, Pierce Chemical Co.) in 10 µl methylene chloride was added. The solvent was evaporated by hand warming to leave a thin coating of iodogen on the vessel walls. Five nmol of tyramine-cellobiose (in 20 µl of 0.15 M sodium phosphate buffer, pH 7.4) was added and gently mixed. Carrier-free $Na^{125}I$ at approximately 500 mCi/ml (Amersham International plc) was added, and iodination was allowed to proceed for 30 min. The reaction was stopped by addition of a mixture of 10 µl 0.1 M NaI and 20 µl 0.1 M $Na_2S_2O_5$ in H_2O.

Activation and protein binding of radioiodinated tyramine-cellobiose

To the solution of ^{125}I-tyramine cellobiose (^{125}I-TC) prepared above, 5 nmol of cyanuric chloride was added in 40 µl of acetone. The reaction was allowed to proceed for 30 sec before addition of the protein for binding. Prior to binding, nucleosomes were dialysed out of 10 mM Tris-HCl and into 0.15 M sodium phosphate buffer, pH 7.4 at 4°C using benzoylated dialysis tubing (Sigma-Aldrich, Poole, Dorset). Five hundred µg (70 µl) of nucleosomes were added to the solution of ^{125}I-TC activated with cyanuric chloride. Binding was carried out for 3 h at room temperature before separation from unbound label by gel filtration on a Sephadex G25 column (PD10, Pharmacia, Milton Keynes, UK) which was equilibrated with TN buffer.

Autoradiography

In order to confirm direct radioiodination of chromatin and nucleosomes and radioiodination of tyramine-cellobiose labelled nucleosome core particles, autoradiography was performed on an 8-18% SDS-PAGE gel onto which the different preparations had been loaded. Three dilutions of each preparation were added, equivalent to approximately 0.3, 0.9 and 1.8 µg of protein. The gel was autoradiographed using Hyperfilm™ in a Hypercassette™ (Amersham International plc) for 8 h before being developed.

Following counting in the gamma counter, dipping film autoradiography was performed on selected samples of mouse liver, spleen and kidney which had been

injected with TC-labelled nucleosomes 24 h previously. Composite blocks of liver, spleen and kidney were created from each of 6 mice (3 SAP-/- and 3 wild-type) and 5 μm sections were cut onto clean slides using a microtome. The slides were dried at 37°C for 24 h, dewaxed, and dipped in K5 nuclear emulsion solution. They were carefully drained and placed onto a cold plate, section uppermost. After 10 min the slides were removed from the cold surface and dried for 3 h. After drying, the slides were placed in a sealed black box, with a small amount of dessicant, and stored at 4°C for 2 wk before development. The slides were developed as follows; in 1:4 solution of LX24 developer in dH_2O for 5 min with constant movement of the slides, washed for 20 sec in dH_2O, fixed for 3 min in a 1:4 solution of Ilford Rapid Fixer in tap water and washed in tap water for a further 5 min. The slides were stained with Mayers Haematoxylin for 5 min before washing in tap water. They were blued in Scott's tap water for 3 min and washed again in tap water. The slides were allowed to dry at 37°C and mounted using DPX mountant. The slides were viewed by light microscopy and labelling was seen as black dots laying over the relevant cell types.

Experimental Protocols

Directly-labelled long chromatin, of which 96% of the activity was TCA precipitable, was injected intra-peritoneally (IP) into 13 female, age-matched C57BL/6 mice from each of 3 genotypes, wild-type, SAP-/- and SAP-/-, hSAP Tg. Each mouse received 2×10^6 counts in 100 μl, equivalent to approximately 10 μg of chromatin. Mice were counted twice in a gamma counter immediately after IP injection and again after 4 h and 7 h.

Preliminary studies of plasma clearance of directly radioiodinated chromatin were performed in 4 wild-type C57BL/6 female mice in which thyroid blockade had been achieved by addition of 60 mg/l potassium iodide to the drinking water for 48 h prior to injection. These studies were undertaken in order to roughly determine the rate of clearance of long chromatin from the blood, so that relevant time points could be selected for comparison of rates of clearance between SAP-/- and wild-type C57BL/6 mice. All mice were injected with 100 μl (100000 counts per min (CPM) corresponding to approximately 1 μg of chromatin) of radioiodinated chromatin into the tail vein. Prior to injection two aliquots of the material were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10% or 2 mM calcium chloride, and the percentage of total counts in each pellet was determined. TCA and calcium precipitability were 98% and 80%, respectively. Approximately 50 μl of blood was obtained from the tail vein 2 min after injection and each mouse was serially bled after a further 15 min, 30 min, and 1, 2, 4 and 5 h. The turnover of chromatin was expressed at each time point as counts per gram of blood as a percentage of counts per gram of blood 2 min after injection (i.e., the counts per gram of blood after 2 min was 100% for each mouse). A single curve was constructed by pooling the information from each mouse at each time point.

Comparison of plasma clearance of long chromatin between SAP-/- and wild-type C57BL/6 mice was performed as follows: for each experiment six female SAP-/- and 6 female, age and weight-matched 6-8 week old wild-type C57BL/6 mice were given potassium iodide for 48 h prior to injection of chromatin in order to provide

thyroid blockade. Each mouse was injected with 100 µl radioiodinated long chromatin and approximately 50 µl of blood was taken exactly 2 min after injection and after a further 15 min, 30 min and 90 min. In order to prevent clotting, all blood was taken directly into tubes which had been left uncapped in a 37°C incubator overnight following addition of 10 µl of 0.2 M EDTA. The blood was microcentrifuged for 5 min and the supernatant (plasma) and pellet were counted separately, confirming that over 96% of the radioactivity was in the plasma. Twenty-five µl of plasma was removed into a 100 µl tube containing 13 µl of 30% TCA. The tubes were microcentrifuged for 5 min and the supernatant was discarded. Each pellet (TCA precipitate) was counted in a gamma counter. Identical experiments, apart from a difference in chromatin dose injected, were performed twice. In the initial experiment approximately 1 µg of chromatin equivalent to 100000 CPM was injected per mouse and for the repeat experiment approximately 5 µg of chromatin per mouse was given (500000 CPM). The same batch of chromatin (also used for the preliminary experiment) was used in both experiments.

Comparison of plasma clearance of nucleosomes between SAP-/- and wild-type C57BL/6 mice was performed as follows: six female SAP-/- and 6 female, age and weight-matched 6-8 week old wild-type C57BL/6 mice were given potassium iodide for 48 h prior to injection of chromatin in order to provide thyroid blockade. Each mouse was injected with 100 µl of directly radioiodinated nucleosome preparation (approximately 1 µg of nucleosomes, 400000 CPM) into the tail vein and was

serially bled after 2 min and a further 15, 30 and 90 min. The blood was collected into EDTA coated tubes, centrifuged and 25 μl aliquots of plasma were precipitated with TCA, prior to counting of the pellet alone.

In order to prove that the observed difference in plasma clearance rate of nucleosomes between SAP-/- and wild-type mice was due specifically to SAP, clearance experiments were repeated comparing 4 groups of mice. There were 5 weight-matched female mice per group and the groups were as follows: wild-type, SAP-/-, SAP-/- after administration of mouse SAP, and SAP-/- after administration of human SAP. One ml of TN buffer alone (as a control) was given intraperitoneally to each mouse from the first two groups 30 min prior to injection of nucleosomes. The latter two groups received 300 μg of mouse SAP in 1 ml of TN buffer and 300 μg of human SAP in 1 ml of TN buffer respectively, given intraperitoneally to each mouse 30 min prior to injection of nucleosomes. As before, mice were bled into EDTA-coated tubes 2 min after injection of nucleosomes and 15 min later, and TCA precipitates were counted.

In order to determine whether the trapped catabolism method was biologically applicable, plasma clearance of TC-labelled nucleosomes was compared with directly radioiodinated nucleosomes by injection of 6 female wild-type mice, following thyroid blockade. Three mice received 100 μl TC-labelled nucleosomes (TCA precipitability 74%) and three age and weight-matched mice received 100 μl directly radioiodinated nucleosomes (TCA precipitability 91%). The plasma decay

kinetics were compared in the two groups by serial bleeds as before. Since only 74% of the tracer in the TC-labelled nucleosome preparation was associated with protein, counting was performed on TCA precipitated material alone.

In order to compare nucleosome catabolism between SAP-/- and wild-type C57BL/6 mice, organ localisation was examined at two different time points. Organ localisation of TC-labelled nucleosomes was examined 24 h after injection in both mouse genotypes in order to determine whether SAP has an effect on overall contribution to catabolism by each organ. In addition, organ localisation of TC-labelled nucleosomes was compared in SAP-/- and wild-type mice 15 min after injection, to determine whether SAP influenced the site of catabolism at the time point at which it influenced clearance of nucleosomes from the plasma.

Several separate experiments were performed. In each experiment, 100 µl TC-labelled nucleosomes (400000 CPM per mouse, as before) were injected into the tail veins of 8 female wild-type and 8 female age and weight-matched SAP-/- mice that had received thyroid blockade, as before. In the initial experiment the mice were anaesthetised 24 h after injection and bled out completely. In two subsequent experiments (performed identically), the mice were anaesthetised and bled out 15 min after injection. In each experiment, the following organs were dissected out in their entirety and transferred into individual tubes containing 1 ml of 10% buffered formalin; liver, spleen, kidneys, heart, lungs, stomach, intestines and adrenal glands. All organs were drained of blood as completely as possible and

stomach and intestines were cleared of food/debris prior to collection. Each organ as well as the blood and carcass of each animal was counted in a gamma counter. The counts per organ were expressed as a percentage of the total number of counts retrieved per animal.

Binding of Pentraxins to Apoptotic Cells

Cell culture and induction of apoptosis

Jurkhat cells were obtained from Dr M Botto, Department of Rheumatology, Hammersmith Hospital and cultured at a cell density of between 2 x 10^5 and 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin and streptomycin. Apoptosis was induced in different experiments, either by ultraviolet (UV) irradiation or by addition of an anti-Fas monoclonal antibody. The time course of development of apoptosis was determined for each method.

UV irradiation was performed as follows; a Petri dish containing 10 ml of cell suspension was placed 2 cm above a UV (312 nm) transilluminator (Ultraviolet Products Ltd, Cambridge, UK) for 10 min at 37°C, before being returned to a standard incubator. In all cases, a control "non-apoptotic" population of cells were placed alongside the UV irradiated cells on a UV shield and subsequently treated identically to the apoptotic cells. Cell viability was determined by trypan blue exclusion at different time points after UV irradiation and binding experiments were performed only on suspensions in which > 95% of cells excluded trypan blue. UV

irradiation of Jurkhat cells for ≥ 20 min resulted in < 95% trypan blue exclusion within 2 h of irradiation and for ≤ 10 min was insufficient for apoptosis induction. Induction of apoptosis with anti-Fas monoclonal antibody, CH11 (Immunotech, Marseilles, France), was by incubation with 50 ng/ml of antibody. In all cases, a control "non-apoptotic" population of cells was incubated in the absence of anti-Fas monoclonal antibody and subsequently treated identically to the apoptotic cells.

Detection of apoptosis and distinction between apoptosis and necrosis

Apoptosis was detected by several methods including DNA laddering, annexin V binding, TdT-mediated uridine nick-end labelling (TUNEL staining) and light microscopy. Once the different methods had been compared, annexin V staining was used most frequently to determine populations of cells undergoing "early" apoptosis. Early apoptotic cells were distinguished from late apoptotic or necrotic cells by trypan blue or propidium iodide exclusion.

Annexin V staining was performed as follows: 1 x 10^5 cells were removed from the cell culture flask, centrifuged at 150 g and washed in cold HBSS. The cells were centrifuged at 150 g, the supernatant was discarded, and they were resuspended in 100 µl of cold "binding buffer." Cells were then incubated for 10 min in the dark with 1 µl of FITC-annexin V +/- 5 µl propidium iodide. After 10 min, a further 400 µl of binding buffer was added to the cell suspension and apoptosis determined by reading on a flow cytometer (Beckman-Coulter). In cases where annexin V staining was not accompanied by incubation of cells with propidium iodide, cells

were stained with trypan blue and the percentage of cells excluding trypan blue was determined.

TUNEL staining was performed using the FragEL apoptosis detection kit. Following incubation with the source of SAP or CRP and staining with fluorochrome-labelled anti-SAP or anti-CRP antibody, cells were cytospun onto a PLL coated slide using a Shandon Cytospin 3, allowed to dry, and fixed in 100% ethanol for 2 min. Slides were immersed in TBS buffer for 15 min at room temperature and then dried. The cells were permeabilised with 100 µl of proteinase K at 20 µg/ml in 10 mM Tris, pH 8 for 5 min and washed in TBS. Each slide was incubated for 20 min with 100 µl of TdT equilibration buffer, blotted, and the cells were coated in 3 µl of TdT enzyme diluted in 57 µl of fluorescein-TdT labelling reaction mix. The cells were covered with parafilm and incubated at 37°C for 90 min, before being washed 3 times in TBS at RT and counterstained with 2 µl of DAPI-containing mounting medium. A coverslip was applied before viewing by confocal microscopy. For each experiment, a positive and negative control was included which consisted of a slide containing known apoptotic cells (HL-60 cells) and a slide to which the TdT enzyme was not applied, respectively.

For detection of apoptotic cells by simple light microscopy, cells were washed in fresh RPMI medium and 100 µl of cell suspension was cytospun onto a slide at 500 rpm for 5 min. The slide was air dried for 30 min before fixation in 100% ethanol and staining with filtered haematoxylin for 30 sec, washing in water for

5 min, and staining in 1% eosin for 2 min. The slides were washed for a further 2 min in tap water, dehydrated and mounted in the usual fashion, before viewing by light microscopy. Small cells with condensed, multi-lobed nuclei were identified as apoptotic.

Pentraxin binding to apoptotic Jurkhat cells in suspension

Jurkhat cells that had been UV irradiated were allowed to proceed for 6 h before binding studies were performed, since exclusion of trypan blue (confirming viability) and presence of apoptosis were present in over 95% and 80% of cells respectively, at this time point. Similarly, 6 h after incubation with a monoclonal anti-Fas antibody, 95% of Jurkhat cells excluded trypan blue and 88% were annexin V positive. Following confirmation of apoptosis, cells were maintained at 4°C to prevent progression to cell necrosis. Cells (3×10^6) were washed twice in cold HBSS, were resuspended and incubated for 30 min at 4°C in either normal human serum (NHS) containing a CRP concentration of 0.5 mg/l and SAP concentration of 26 mg/l, acute phase human serum (APHS) containing a CRP concentration of 92 mg/l and SAP concentration of 51 mg/l, or pure SAP at 150 mg/l in TCB buffer containing 4% BSA and pure CRP at 1000 mg/l in TC. In all binding experiments, 3×10^6 control cells were simultaneously incubated with the appropriate source of SAP and/or CRP in the presence of 10 mM EDTA in order to chelate calcium. Specific ligand-binding by pentraxins is calcium dependent, and this property was used as a specificity control. After incubation for 30 min, cells were washed 3 times in 400 μl of TCB buffer containing 1% BSA (cells incubated in

the presence of EDTA were washed in TEB buffer containing 1% BSA) before staining with fluorescently labelled anti-human SAP or anti-human CRP antibodies.

Staining with fluorochrome labelled anti-human SAP and anti-human CRP

The IgG1 fraction of a sheep anti-human SAP antiserum, raised by immunisation with isolated, pure human SAP was fluorescently labelled with tetramethylrhodamine isothiocyanate (RITC) as follows. The IgG at 40 mg/ml in PBS was dialysed overnight at 4°C into 0.5 M sodium carbonate buffer (CB), pH 9.5. RITC (mixed isomers) was diluted to 2 mg/ml in CB and added dropwise to the IgG while gently stirring at a ratio of 40 μg:1 mg IgG and stirred overnight at 4°C. The free fluorochrome was separated from coupled IgG by gel filtration on a Sephadex G25 column (PD10, Pharmacia, Milton Keynes, UK) which was equilibrated with CB. The IgG conjugate was dialysed overnight at 4°C into 0.0175 M phosphate buffer (PB), pH 6.3, and was fractionated by ion exchange chromatography using DE52 cellulose (Whatman International Ltd, Maidstone, UK) which had been equilibrated for pH and conductivity with PB. Fractions were eluted stepwise with PB containing 0.125 M, 0.25 M and 0.5 M NaCl. The fluorochrome:protein (F:P) ratio and protein concentration were determined for each fraction by reading the optical density at A_{280} and A_{515} using the Beckmann DU650 spectrophotometer, and a suitable fraction was selected. The F:P ratio and protein concentration of the selected fraction were 2.2:1 and 0.46 mg/ml, respectively. Before use, the conjugated rhodamine-anti-human SAP was dialysed against PBS containing 0.1% sodium azide.

Specific binding of the RITC-anti-human SAP to CNBr-Sepharose beads to which isolated human SAP or CRP had been covalently coupled at 1 mg protein per ml beads was used to test the antibody conjugate and determine the appropriate dilution for use, with Sepharose beads bearing CRP as the negative control. This was performed as follows: sedimented human SAP and CRP beads were diluted 1:10 in TC buffer. The RITC-anti-human SAP was diluted 1:2, 1:5, 1:10, 1:20, 1:50, 1:100 and 1:200 in TC buffer and 50 µl aliquots of each dilution were mixed separately, with 50 µl of SAP and CRP bead suspensions, and incubated in the dark for 30 min at 37°C with occasional agitation. After 30 min, the mixtures were centrifuged for 5 min and the supernatants were discarded. The beads were washed 3 times in 1 ml of TC buffer, resuspended in 20 µl of TC buffer, and viewed under a fluorescence microscope. There was no staining of the beads bearing CRP at any dilution. The beads bearing SAP were stained at antibody dilutions of 1:2, 1:5 and 1:10 but staining disappeared at dilutions above 1:10. Since there was little background at 1:5 but excellent fluorescence of the beads, this dilution was selected for optimal staining of apoptotic cells.

Fluorescein-labelled sheep anti-human SAP (FITC-anti-human SAP), that had been prepared and tested by the same methods, was available in the laboratory.

The IgG fraction of a goat anti-human CRP antiserum, raised by immunisation with isolated pure human CRP, was fluorescently labelled with fluorescein (FITC) as follows. The IgG was dialysed overnight at 4°C into 0.1 M sodium phosphate

buffer, pH 9.5. Fluorescein isothiocyanate (isomer 1) was diluted to 2 mg/ml in 0.1 M phosphate buffer and added dropwise to the IgG while gently stirring at a ratio of 50 μg:1 mg IgG and stirred overnight at 4°C. The free fluorochrome was separated from coupled IgG by gel filtration on a Sephadex G25 column (PD10, Pharmacia, Milton Keynes, UK) which was equilibrated with 0.1 M phosphate buffer. The FITC-IgG conjugate was dialysed overnight at 4°C into 0.01 M phosphate buffer, pH 7.6, and was fractionated by ion exchange chromatography using DE52 cellulose (Whatman International Ltd, Maidstone, UK) which had been equilibrated for pH and conductivity with 0.01 M phosphate buffer. Fractions were eluted stepwise with 0.01 M phosphate buffer containing 0.2%, 0.4%, 0.8%, 1%, 2%, 5% and 0.4 M phosphate buffer. The fluorochrome:protein (F:P) ratio and protein concentration were determined for each fraction by reading the optical density at A_{280} and A_{495} using the Beckmann DU650 spectrophotometer, and a suitable fraction was selected. The F:P ratio and protein concentration of the selected fraction were 3.5:1 and 2.73 mg/ml, respectively. Before use, the conjugated FITC-anti-human CRP was dialysed against PBS containing 0.1% sodium azide.

Specific binding of the FITC-anti-human CRP to Sepharose beads bearing human CRP was used to test the antibody conjugate and determine the appropriate dilution for use, with Sepharose beads bearing SAP as the negative control. The method was identical to that for the RITC-anti-human SAP. Staining of the beads bearing SAP was absent at all dilutions but staining of beads bearing CRP was present at 1:2, 1:5,

1:10 and 1:20 but staining disappeared at dilutions above 1:20. Since there was little background at 1:10 but excellent fluorescence of the beads, this dilution was selected for optimal staining of apoptotic cells.

Suspensions of apoptotic cells (3×10^6 cells per sample) on ice were stained by incubation in the dark for 30 min in 25 µl per sample of a 1:5 dilution in TC buffer of RITC-anti-human SAP, or a 1:10 dilution in TC buffer of FITC-anti-human CRP, or a 1:50 dilution in TC buffer of FITC-anti-human SAP. After 30 min, the cells were washed 3 times in 400 µl of TC buffer before being examined and counted by microscopy and/or flow cytometry. In all experiments, a separate control sample of apoptotic cells was simultaneously incubated with antibody which had previously been absorbed with the respective immobilised pure antigen. This was performed as follows: sedimented beads bearing the pure antigen were incubated with the fluorescent antibody at an antigen:antibody ratio of > 10:1 for 30 min at 37°C and then overnight at 4°C with constant rotation. The beads were centrifuged and the supernatant was removed and diluted to the required dilution in TC. Cells were incubated with 25 µl of the appropriately diluted supernatant as a negative control in order to demonstrate specificity of the fluorochrome-labelled antibody for the desired antigen.

Competition between SAP and CRP for ligands on the surface of apoptotic cells

In order to determine whether there is competition between SAP and CRP for ligands on the surface of apoptotic cells, SAP binding was determined in the presence of

increasing concentrations of CRP, and CRP binding was determined in the presence of increasing concentrations of SAP. Apoptosis was induced in Jurkhat cells by incubation for 6 h with anti-Fas monoclonal antibody, as before. For each experiment a total of seven samples each containing 3×10^6 apoptotic cells, were incubated at 4°C for 30 min with different concentrations of SAP and CRP, and SAP and/or CRP binding was determined, as before.

When examining SAP binding, a single sample of 3×10^6 cells was incubated with SAP (at a concentration of 150 mg/l in TCB containing 4% BSA) in the absence of CRP. Each of five further samples of cells (3×10^6 cells per sample) were incubated with NHS containing SAP at a concentration of 26 mg/l and the following concentrations of CRP respectively; 0.5 mg/l, 1 mg/l, 10 mg/l, 100 mg/l and 1000 mg/l. As a negative control, SAP binding was determined after incubation of apoptotic cells with CRP alone in TC at 1000 mg/l (in the absence of SAP).

When examining CRP binding, several separate samples each containing 3×10^6 apoptotic Jurkhat cells were incubated for 30 min in 50 µl of human serum containing CRP at a concentration of 50 mg/l and the following concentrations of SAP respectively; 0.5 mg/l, 1 mg/l, 10 mg/l, 100 mg/l and 150 mg/l. As a negative control, CRP binding was determined after incubation of apoptotic cells with SAP alone in TCB at 150 mg/l (in the absence of CRP).

Immune Response to Administration of Syngeneic Apoptotic Thymocytes in vivo

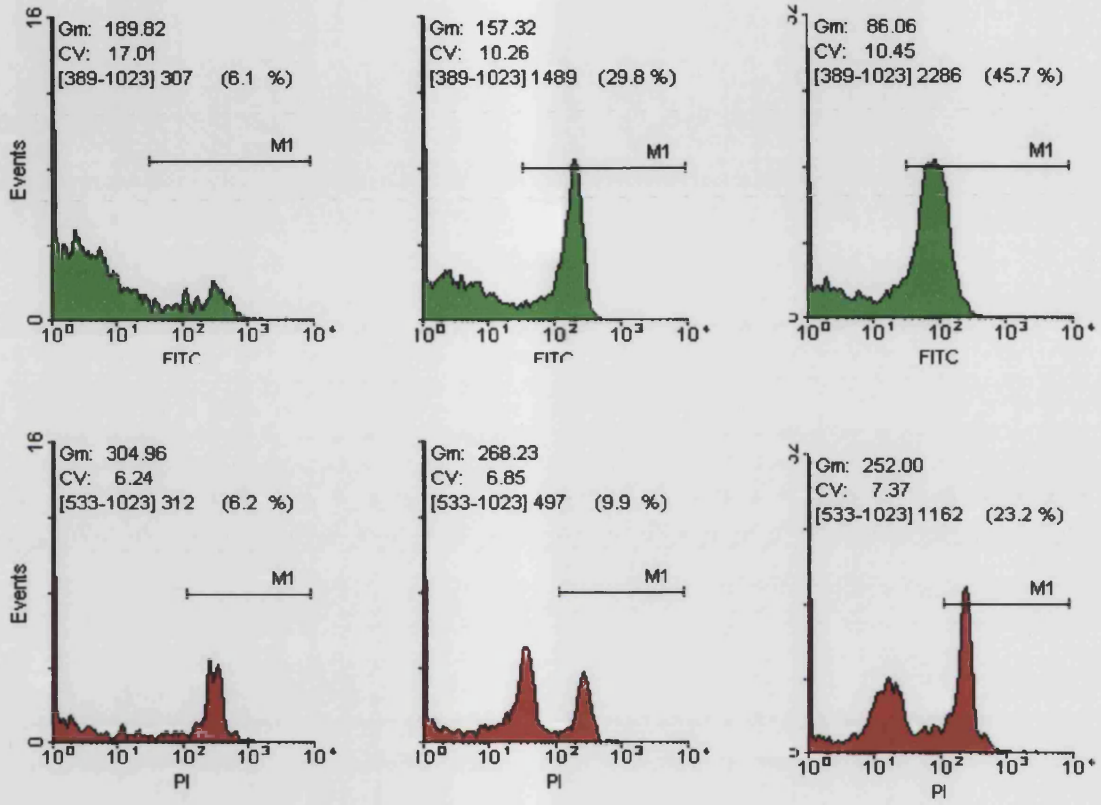
Thymocyte extraction and apoptosis induction

Thymocytes were obtained from 6-8 week old SAP-/- mice of the inbred strain to be injected. Mice were killed by CO_2 inhalation and the thymus gland was immediately exposed, excised whole and transferred into 5 ml of serum-free RPMI medium on ice. Assuming a yield of 1 x 10^8 thymocytes per mouse, the required number of thymus glands were then transferred into 10 ml per thymus of fresh ice cold serum-free medium in a class II flow cabinet (Envair Ltd, Rossendale, UK). Each thymus gland was gently crushed with as little shearing as possible, and the cell suspension was pipetted through a tea strainer into a sterile tube. The cell density was determined using a cytometer and the cells were centrifuged at 150 g at 4°C for 5 min. The supernatant was discarded and the cells were resuspended in fresh serum-free RPMI medium at a density of 1 x 10^7 cells/ml and transferred to a cell culture flask. The thymocytes were then cultured in an incubator at 37°C containing 10% CO_2 for 6-12 h (until detection of apoptosis). Apoptosis was initially determined by several methods, as before (figure 2.2). Once these had been compared, annexin V staining alone was used since this was found to be an early indicator of apoptosis, particularly in the absence of coexistent staining with propidium iodide or trypan blue. Annexin V staining was performed as before.

Intravenous administration of apoptotic cells

Apoptotic thymocytes were centrifuged at 150 g for 5 min at 4°C, the supernatant was discarded and the cells resuspended at a density of 1 x 10^8 cells/ml in cold,

A)



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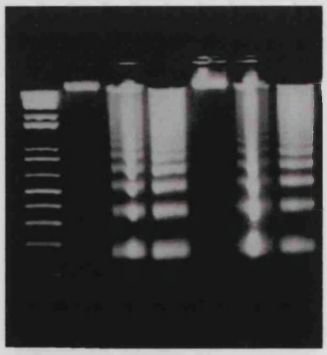

M 0 4 8 0 4 8

Figure 2.2 Confirmation of thymocyte apoptosis by A) flow cytometry and B) agarose gel electrophoresis A) Annexin V (green) and propidium iodide (red) staining at T=0 (left) T=4 hours (middle) and T=8 hours (right) after removal from the animal and culture in serum-free medium. B) Characteristic DNA laddering associated with apoptosis after *ex vivo* culture of thymocytes for 4 and 8 hours. There was no laddering at T=0 hours. The lane on the left (M) is a control sample containing DNA fragments of repeating lengths of 1 kb to a maximum of 12 kb.

sterile PBS buffer. A 100 µl aliquot of the cell suspension (1 x 10^8 cells/ml) was injected into the tail vein of each mouse weekly for 4 weeks. Non-apoptotic thymocytes were obtained for intravenous injection using the same method, but were re-suspended in cold PBS for injection without prior incubation. Mice were bled prior to the first injection and 2, 4, 6 and 8 weeks later, at which time they were sacrificed and the kidneys obtained for renal histology.

All sera were tested for ANAs, and sera from day 28 were additionally screened for antibodies to dsDNA, chromatin and ssDNA. Kidneys were fixed in Bouin's solution, stained with PAS and viewed blind by Professor HT Cook, as before.

For comparison of the autoimmune response between SAP-/- and wild-type C57BL/6 mice, results from 2 identical experiments were pooled. Importantly, the mice in both experiments were the same age at the time they received their first intravenous injection (Day 0), and the experimental protocol was identical in each experiment. In total, 26 female and 9 male SAP-/- C57BL/6 mice and 20 female and 10 male age and weight-matched wild-type C57BL/6 mice were injected.

For comparison of the autoimmune response between SAP-/-, wild-type and SAP-/-, human SAP transgenic C57BL/6 mice, each of 16 female SAP-/- mice, 10 female wild-type and 16 female SAP-/-, human SAP transgenic mice were injected with apoptotic thymocytes, as before.

For comparison of the immune response between SAP-/- and wild-type 129/Sv mice, each of 17 (9 males and 8 females) SAP-/- mice and 17 (9 males and 8 females) wild-type mice, were injected with apoptotic thymocytes, as before.

Macrophage Ingestion of Apoptotic Mouse Thymocytes in vivo

Thymocytes were obtained from SAP-/- or wild-type 6-8 week old C57BL/6 mice as before. In order to speed development of apoptosis, 2 μM dexamethasone was added to the serum-free RPMI culture medium containing the thymocytes. Apoptosis was detected by flow cytometry after 4 h by staining with annexin V and propidium iodide, as before (figure 2.3).

For each experiment, 6 female SAP-/- and 6 female age- and weight-matched wild-type mice were injected intraperitoneally with 1 ml of sterile 3% thioglycollate to induce sterile peritonitis. Three days later, mice were injected intraperitoneally with 2×10^7 apoptotic murine thymocytes in 200 μl of PBS buffer. In all experiments, mice were killed after 30 min and peritoneal cells were recovered by lavage with 6 ml of ice cold HBSS. The 30 min time point was based on comparable experiments reported by Taylor and colleagues in which the full time course was studied [279]. The cells were washed in serum-free RPMI medium and further diluted to a final concentration of 1×10^5 cells/ml, before being cytospun onto a slide and air dried for 30 min. The specimens were fixed in 100% methanol for 10 min before immersion in May-Grünwald stain 0.3% w/v for 10 min. They were then stained in 10% Giemsa in Sorensens buffer for 15 min and washed in

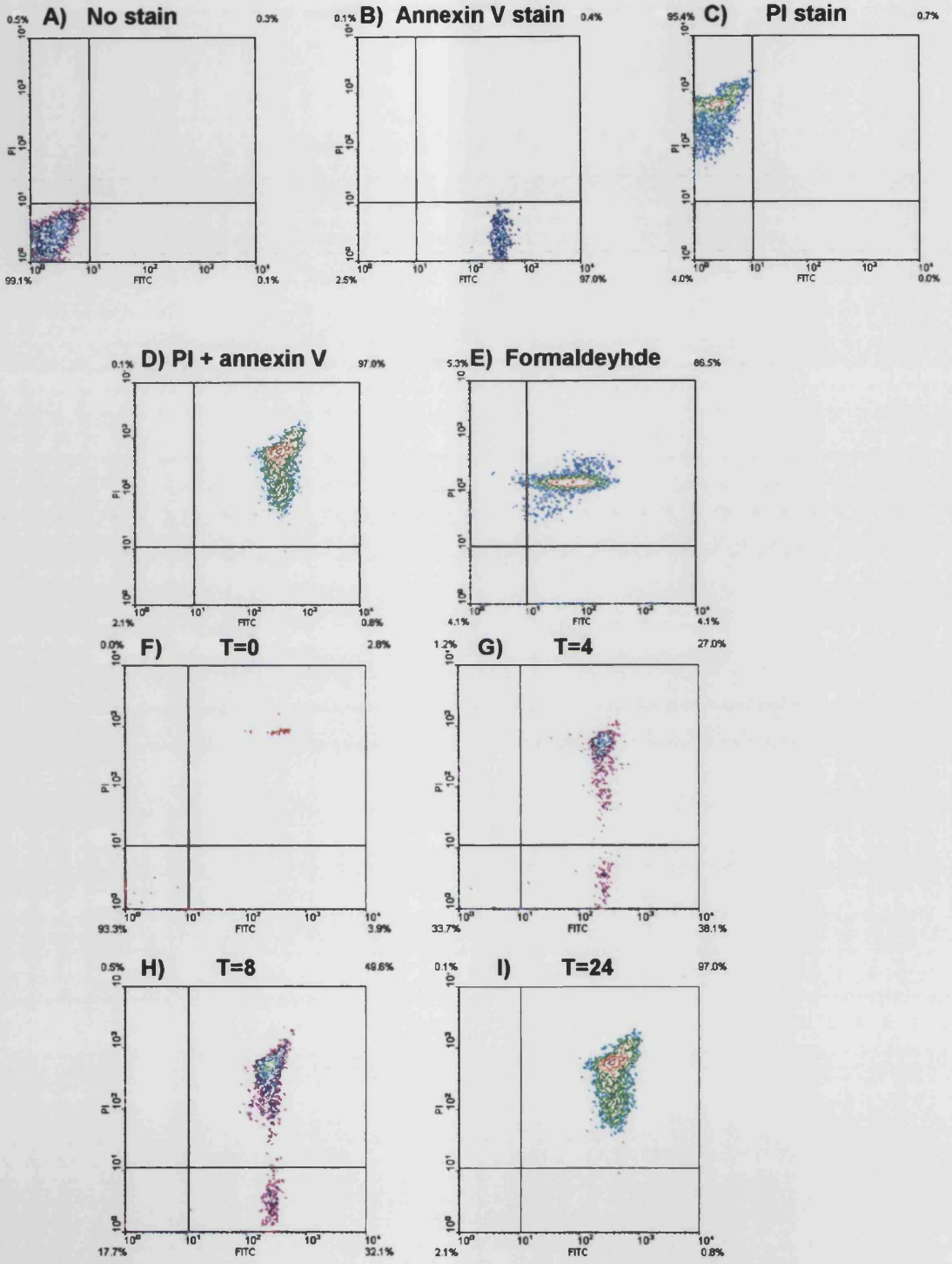

Figure 2.3 Time course of apoptosis induction in mouse thymocytes after incubation with 2 μM dexamethasone. Staining with annexin V and propidium iodide are shown on the x and y axis, respectively. A-E) Staining of late apoptotic cells demonstrating flow cytometer settings A) Absence of signal in the absence of staining B) Annexin V signal alone and C) PI signal alone, when stained with each reagent, respectively D) Staining of late apoptotic cells and E) Staining of cells exposed to formaldehyde, with both Annexin V and PI F-I) Time course of apoptosis showing that after 4 hours of incubation with 2 μM dexamethasone, 38% of cells were annexin V positive and PI negative (early apoptotic) and 27% were positive for both (late apoptotic).

Sorensens buffer, pH 6.8 for 4 min before being air dried and mounted in DPX mountant. Slides were viewed by brightfield light microscopy using the Leica CTR Mic. Macrophages were easily recognisable and distinguishable from lymphocytes. Phagocytosis was scored on coded cytospins of stained cells, and was expressed as the percentage of macrophages ingesting apoptotic cells. Apoptotic cells were considered phagocytosed when > 50% of the cell volume was contained within the border of the macrophage, although this may underestimate the level of phagocytosis [335]. Between 200 and 300 macrophages were scored in each sample. Three identical replicate experiments were performed with injection of SAP-/- apoptotic thymocytes and wild-type apoptotic thymocytes injected into each genotype, respectively.

Macrophage Ingestion of Apoptotic Human Lymphocytes in vitro

Peripheral blood mononuclear cells were isolated from healthy donors as follows. Venous blood (30 ml) was taken into citrated tubes, and 5 ml volumes were gently layered onto 4 ml volumes of *Lymphoprep*^{TM} (Nycomed Pharma, Oslo, Norway) in 15 ml plastic centrifuge tubes. The tubes were centrifuged at 800 g for 20 min at 4°C and the interface containing lymphocytes and monocytes was removed and inserted into a single 50 ml tube. Cold, sterile PBS was added to a final volume of 50 ml and the cells were centrifuged at 350 g for 10 min. The supernatant was discarded and the cells were washed with 25 ml fresh PBS before centrifugation and removal of the supernatant. Cells were re-suspended in 5 ml of cold DMEM medium, counted and diluted in further DMEM to a final concentration of

4×10^6 cells/ml. A sterile 13 mm glass coverslip was added to the base of each well of a 24-well plate and 200 µl of the cell suspension was pipetted onto each coverslip. The cells were incubated for 2 h at 37°C and washed 3 times with 1 ml per well of fresh, warm DMEM medium to remove any non-adherent cells. Differentiated, elongated macrophages were ready for use after 7 days of culture in DMEM medium.

Jurkhat cell apoptosis was induced by culturing cells in the presence of anti-Fas monoclonal antibody as described earlier. Apoptosis was confirmed by annexin V binding and cells were incubated with human serum as a source of SAP as described earlier. For comparison, cells were incubated with SAP in the presence of 10 mM EDTA. Binding of SAP (and absence of SAP binding in the samples incubated in the presence of EDTA) was confirmed by incubation of cells with anti-human SAP and microscopy as described earlier.

SAP-coated apoptotic Jurkhat cells (and apoptotic cells not coated in SAP for comparison) were re-suspended in serum-free DMEM containing 2 mM calcium chloride at 1.5×10^7 cells/ml, and 200 µl of the cell suspension was incubated for 1 h at 37°C with each well of washed macrophages. After 1 h the coverslips were removed from each well into a histology cassette, washed 3 times in cold PBS, and stained with Giemsa overnight before dehydration and mounting, cell side down, onto a slide.

Slides were viewed blind and results were expressed as the percentage of macrophages ingesting apoptotic cells. In each sample between 200 and 300 macrophages were counted. Apoptotic cells were considered phagocytosed when more than 50% was contained within the border of the macrophage. Three identical replicate experiments were performed, although an additional negative control consisting of incubation of macrophages with non-apoptotic Jurkhat cells was performed in a single experiment, as well as the standard negative control of incubation of macrophages in the absence of Jurkhat cells.

Chapter 3 – Spontaneous Phenotype Of SAP Knockout Mice

INTRODUCTION

Chromatin and other nucleic acid-protein complexes are primary targets of pathogenic autoantibodies in the prototypic systemic autoimmune disease systemic lupus erythematosus (SLE). There are several lines of evidence to suggest that the autoimmune response in SLE is antigen driven which include association of disease with particular MHC class II alleles [260,264], the nature of the autoantibodies in SLE [261,262], and the abrogation of disease in mouse models with a variety of antibodies that interfere with T cell interactions [263,265-267]. Native DNA is poorly immunogenic and DNA-proteins complexes are now believed to be the major immunogen in SLE [269-272,274].

Nucleosomes and chromatin are released into the circulation by apoptotic cell death; however, little is known about the mechanisms of their clearance. Recent evidence suggests that the pentraxin family of plasma proteins may have an important *in vivo* role in clearance of nucleosomes and chromatin.

Serum amyloid P component (SAP) is the major calcium-dependent specific DNA binding protein in the serum [60], and, under physiological conditions, solubilises native long chromatin by binding to DNA and displacing histone H1 [61]. SAP also

binds to exposed chromatin *in vivo,* for example in skin lesions of patients with SLE [299], and to dermal keratin bodies, which are apoptotic keratinocytes, in normal skin [300]. It has been claimed that CRP also binds to chromatin, but as originally reported by Du Clos [307], CRP from whole serum binds exclusively to small nuclear ribonucleoprotein particles under physiological conditions *in vitro*, and presumably also *in vi*vo [62].

In a number of autoimmune diseases, such a rheumatoid arthritis, Crohn's disease and rheumatic fever, CRP concentration is the single most sensitive objective criterion of activity of disease [151,152,315,316]. In marked contrast to most autoimmune disorders, disease activity in SLE with severe symptoms and continuing tissue damage, is associated with CRP levels which are raised only modestly, despite other tests for active inflammation being positive [110,156]. The mechanism for this apparently inappropriate failure of production in SLE is not known. An intriguing possibility is that the capacity to respond to particular forms of inflammation, tissue injury or other stimuli by production of CRP is genetically determined, and that CRP-responder status could contribute to hereditary predisposition to SLE.

Few studies have investigated the role of pentraxin binding to nuclear antigens *in vivo.* In the (NZB x NZW)F_1 female hybrid mouse model of SLE, the autoimmune phenotype was reportedly accelerated by intraperitoneal injections of chromatin, and the effect was abrogated by co-injection of CRP [320]. However the results in this paper do not robustly support the authors' conclusions. The

spontaneous phenotype of mice with targeted deletion of the SAP gene was first reported from our laboratory [232]. A cohort of (129/Sv x C57BL/6)F_2 SAP deficient mice were noted to spontaneously develop antinuclear autoimmunity, including anti-chromatin, anti-histone and anti-DNA antibodies, with 41% of deficient females mice developing immune complex glomerulonephritis. The clearance of administered chromatin was also reported to be faster in SAP deficient animals compared to their wild-type counterparts. However, these studies were performed in an inevitably genetically heterogeneous group of mice, and whether SAP deficiency in pure-line mice is associated with autoimmunity has not been previously reported.

The present results demonstrate that SAP deficiency is indeed importantly associated with development of autoimmunity, but highlight simultaneously, the complexity of the genotype-phenotype relationship in SLE, and its dependence upon the interaction of multiple genetic loci. Pure-line C57BL/6 mice with targeted deletion of the SAP gene spontaneously developed antinuclear autoimmunity and glomerulonephritis, a phenotype closely resembling human SLE. However, SAP deficiency in the 129/Sv, "non-autoimmune" strain of mouse was insufficient to cause the lupus-like phenotype.

SPONTANEOUS DEVELOPMENT OF AUTOIMMUNITY IN PURE-LINE C57BL/6 SAP DEFICIENT MICE

The deleted SAP gene was backcrossed for 6 generations onto the inbred C57BL/6 mouse strain by Dr M Botto, Department of Rheumatology, Imperial College School of Medicine to produce "pure-line" C57BL/6 SAP deficient mice. A cohort of 312 C57BL/6 mice, 154 males (49.4%) and 158 females (50.6%) were followed for 12 months, all housed under identical conditions. One hundred and three (33%) of the mice were wild-type (WT), of which 50 were female and 53 were male; 104 (33.3%) were heterozygous for deletion of the SAP gene (SAP+/-), of which 53 were female and 51 were male; and 105 (33.7%) were homozygous for the SAP gene deletion (SAP-/- or SAP deficient), of which 55 were female and 50 were male.

During the course of follow up 3 wild-type mice were lost, one at < 3 months of age, one between 6 and 9 months and one between 9 and 12 months of age. One wild-type mouse was killed at 10 months due to rectal prolapse. One heterozygous mouse was lost between 6 and 9 months of age, and one was killed at 3 months due to an ear infection. Three SAP-/- mice were lost, one between 3 and 6 months of age, one between 6 and 9 months, and one between 9 and 12 months of age. Two further SAP-/- mice were found dead, one at less than 3 months of age, and one at 7months. Two SAP-/- mice were killed due to skin injuries at 6 and 10 months, and one at 11 months because of an ear infection. All other mice were healthy until they were killed by exsanguination at 12 months of age. Mice killed between 9 and 12 months of age were included in the 12 month analysis.

All available mice were bled from the tail vein at 6 months of age and a representative sample of 215 and 219 mice were bled at 3 and 9 months respectively. All mice were killed by bleed out from the axillary artery at 12 months of age before harvesting the following organs in each case: liver, spleen, kidneys, heart, lungs, salivary gland, aorta, stomach, small intestine, large intestine.

Serological Analysis

Serum obtained at 3, 6, 9 and 12 months was tested for the presence of anti-nuclear antibodies (ANAs). Samples were screened at a dilution of 1/80 and titrated to end-point. ANA staining was always homogeneous. At 3 months of age ANAs were present in 7.6% SAP-/- mice (5 of 66) at a titre of 1/80, and none of 57 SAP+/- mice and 91 wild-type mice ($P = 0.004$, Chi-squared test). Presence and titre of ANAs increased over time in the majority of mice, but particularly in SAP deficient animals (figure 3.1). Information on ANA titres at both 3 months and 12 months was available in 211 mice. In this subgroup, titres increased in 127 (60.2%) and remained the same in 74 (39.8%); no mouse showed a drop in titre over this period ($P = 0.0001$, Sign rank test). By six months of age ANAs were present in 5.9% of wild-type mice (median = 0; range = 0-1/320), 30.1% of SAP+/- mice (median = 0; range = 0-1/640) and 82.8% of SAP-/- mice (median = 0; range = 0-1/640) ($P = 0.001$, Chi squared test) and by 9 months of age 8.6% (median = 0; range = 0-1/1280), 37.5% (median = 0; range = 0-1/640) and 95.7% (median = 1/160; range = 0-1/1280) of wild-type, SAP+/- and SAP-/- mice, respectively ($P = 0.001$).

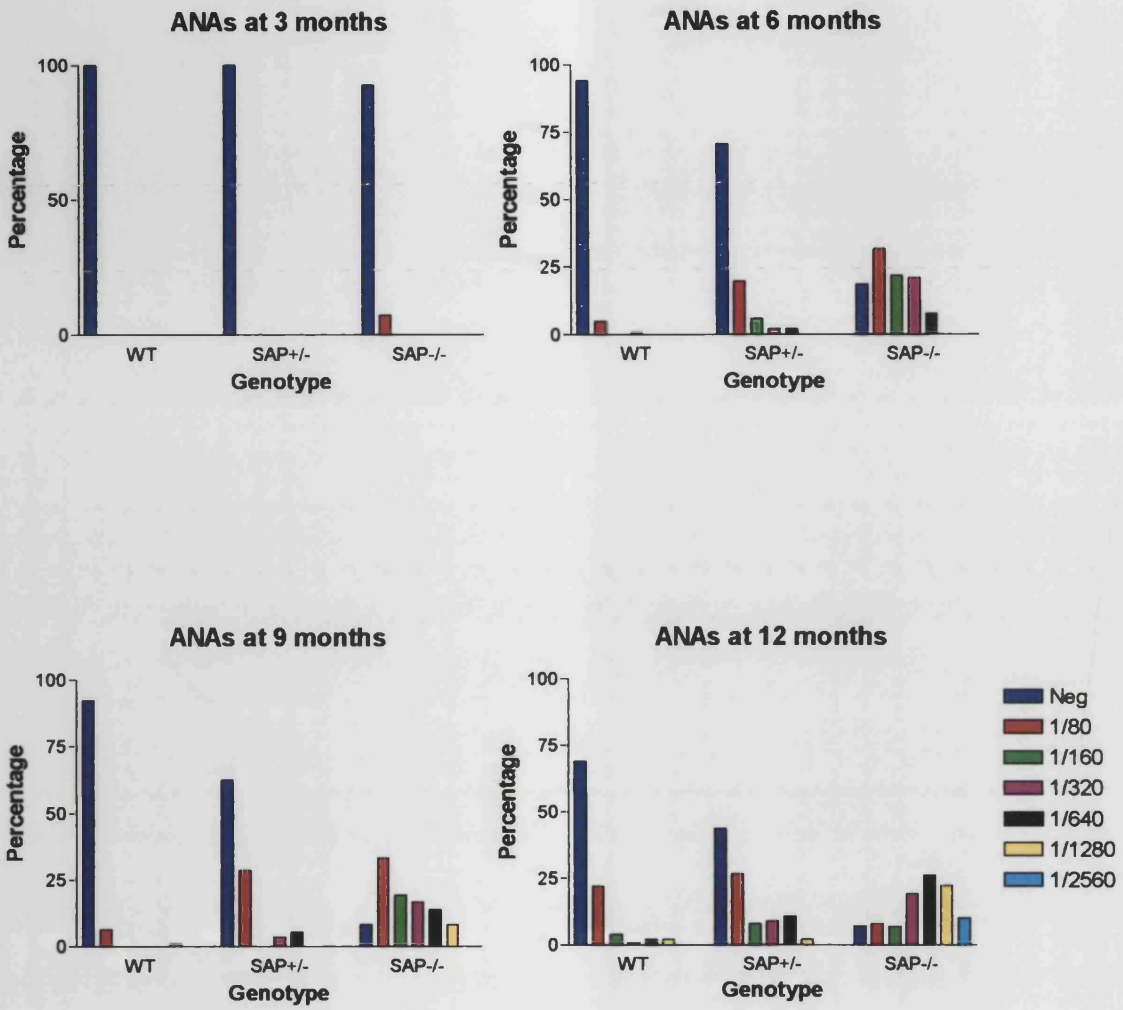

Figure 3.1 Time course of development of anti-nuclear autoantibodies (ANAs) in C57BL/6 mice according to genotype. Presence and titre of ANAs increased over time in the majority of mice, but particularly in SAP deficient animals. Among 211 mice in which ANA titres were determined at both 3 and 12 months of age, titres increased in 127 (60.2%) and remained the same in 74 (39.8%); no mouse experienced a drop in titre over this period (P = 0.0001, Sign rank test).

At 12 months of age ANAs were found in 31% of wild-type mice (median = 0; range = 0-1/1280), 56.9% of SAP+/- mice (median = 1/80; range = 0-1/1280) and 92% of SAP-/- mice (median = 1/640; range = 0-1/2560) (P = 0.001, Chi-squared test) with significantly higher titres among SAP-/- compared to SAP+/- mice, and, in turn, SAP+/- compared to wild-type animals (P = 0.0001, Mann-Whitney U test) (figure 3.2).

At 12 months of age ANAs were detected in 40% (20/50) of female wild-type mice (median = 0; range = 0-1/1280), 64.2% (34/53) of female SAP+/- mice (median = 1/80; range = 0-1/1280) and 88.2% (45/51) of female SAP-/- mice (median = 1/640; range = 0-1/2560) (P = 0.001, Chi-squared test; P = 0.0001, Mann-Whitney U test). ANAs were detected in 22% (11/50) of male wild-type mice (median = 0; range = 0-1/640), 49% (24/49) of male SAP+/- mice (median = 0; range = 0-1/1280) and 97.9% (47/48) of male SAP-/- mice (median = 1/640; range = 0-1/2560) (P = 0.001, Chi-squared test; P = 0.0001, Mann-Whitney U test) (figure 3.3).

The remaining serological analyses were carried out only on serum obtained at 12 months of age.

Anti-dsDNA antibodies were analysed by indirect immunofluorescence using slides coated with *Crithidia luciliae* as a substrate. Samples were screened at a dilution of 1/20 and titrated to end point. Titres of 1/20 or above were considered positive. At

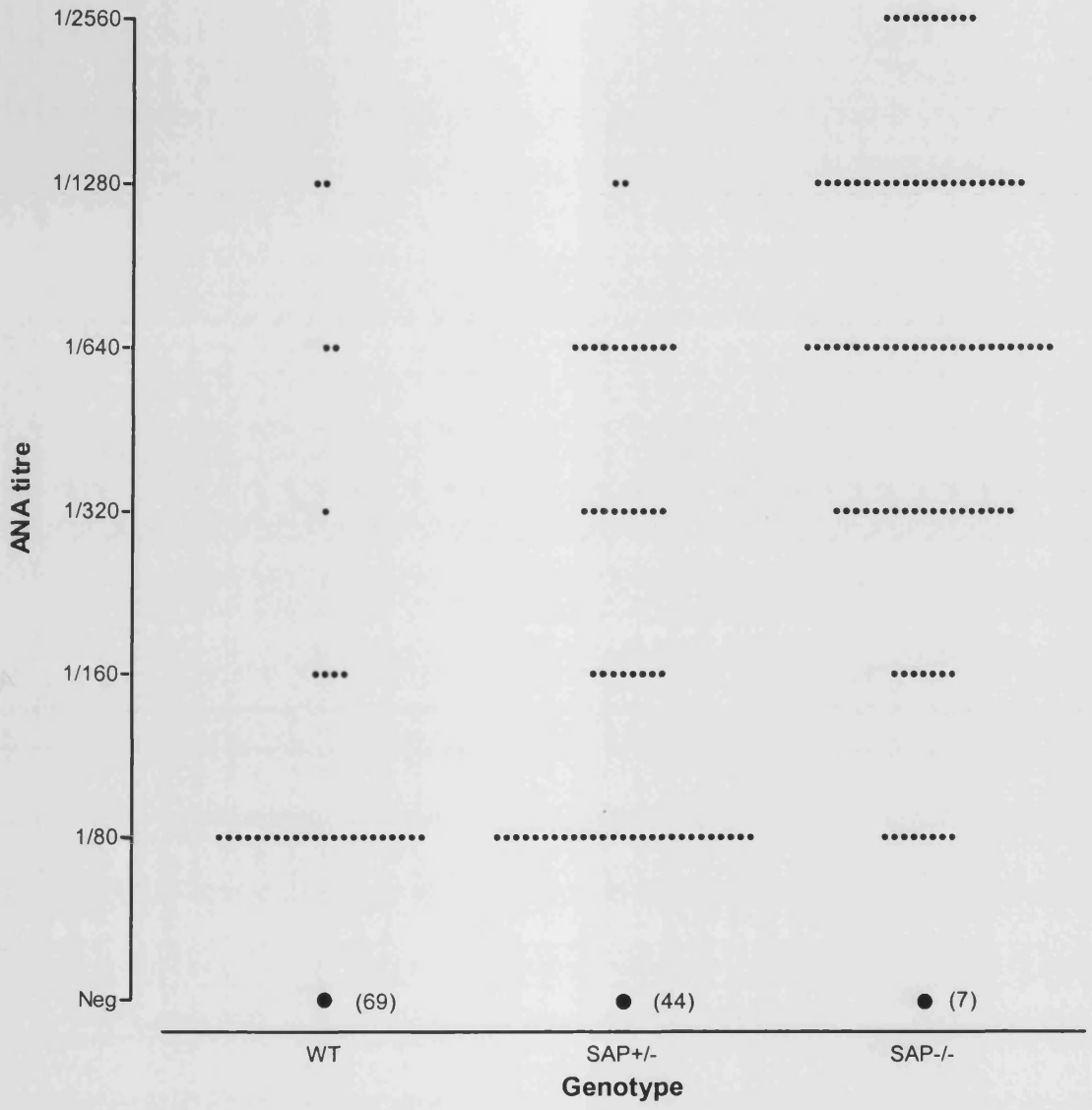

Figure 3.2 Anti-nuclear antibody (ANA) titres at 12 months of age in wild-type (WT) C57BL/6 mice compared to mice heterozygous (SAP+/-) and mice homozygous (SAP-/-) for deletion of the SAP gene. There were more SAP-/- mice with ANAs compared to WT mice, with SAP+/- mice intermediate between them (Chi-squared test, $P<0.001$), and there was a progressive increase in ANA titres from wild-type to SAP+/- to SAP-/- mice (Mann-Whitney U test, $P<0.0001$).

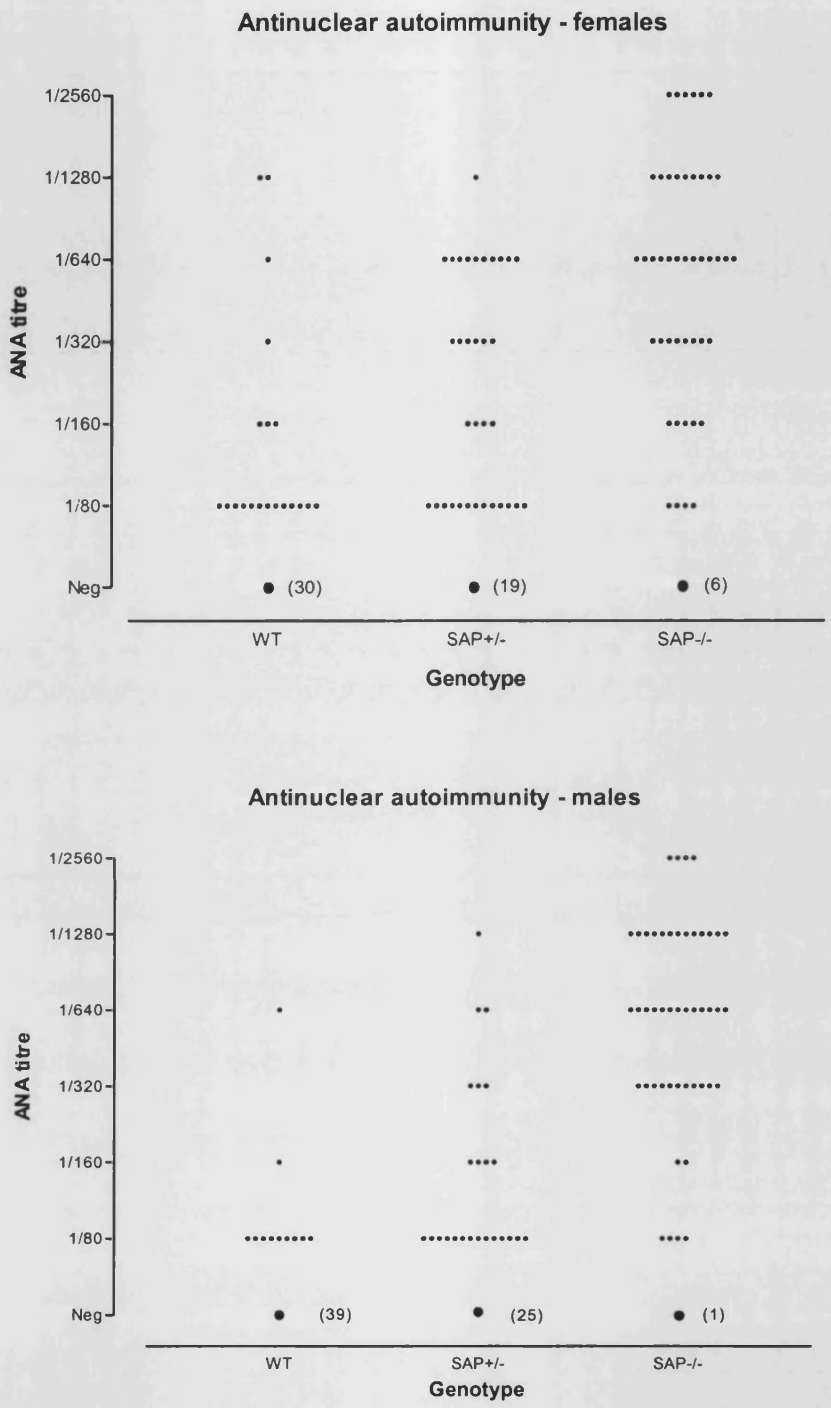

Figure 3.3 Anti-nuclear antibody (ANA) titres at 12 months of age in female and male wild-type (WT) C57BL/6 mice compared to mice heterozygous (SAP+/-) and homozygous (SAP-/-) for deletion of the SAP gene. There were more SAP-/- mice of both sexes with ANAs compared to wild-type mice, with SAP+/- mice intermediate between them (Chi-squared test, P=0.001), and there were higher ANA titres in SAP deficient mice of both sexes compared to SAP+/- and wild-type mice (Mann-Whitney U test, P=0.0001).

12 months of age significantly increased anti-dsDNA antibody titres were seen in 64.7% (33/51) of female SAP-/- mice (median = 1/20; range = 0-1/80), compared to 26.4% (14/53) of female SAP+/- mice (median = 0, range = 0-1/80) and 6% (3/50) of female wild-type mice (median = 0, range = 0-1/80) (P = 0.001, Chi-squared test). Anti-dsDNA antibodies were present in 39.6% (19/48) of male SAP-/- mice (median = 0; range = 0-1/80), compared to 18.4% (9/49) of male SAP+/- mice (median = 0, range = 0-1/80) and 12% (6/50) of male wild-type mice (median = 0, range = 0-1/80) (P = 0.001, Chi-squared test) (figure 3.4).

Antibodies to histones, chromatin and ssDNA were all measured by immunoradiometric assay (IRMA) and results expressed in arbitrary units based on a standard of pooled serum from MRL/Mp-*lpr/lpr* mice, as described in Materials and Methods. The dilutions used in each assay are outlined in Materials and Methods.

Significantly higher titres of anti-histone antibodies were seen in male SAP-/- mice (median = 13.5 units) (figure 3.5) compared to their SAP+/- (median = 8 units) and wild-type (median = 8 units) counterparts (P = 0.01, and P = 0.009 respectively, Mann-Whitney U test). There were no significant differences in anti-histone antibody titres between the three groups of female mice (P = 0.1, Mann-Whitney U test).

Significantly higher titres of anti-chromatin antibodies were seen in male and female SAP-/- mice (males: median = 39 units, range = 0-169; females:

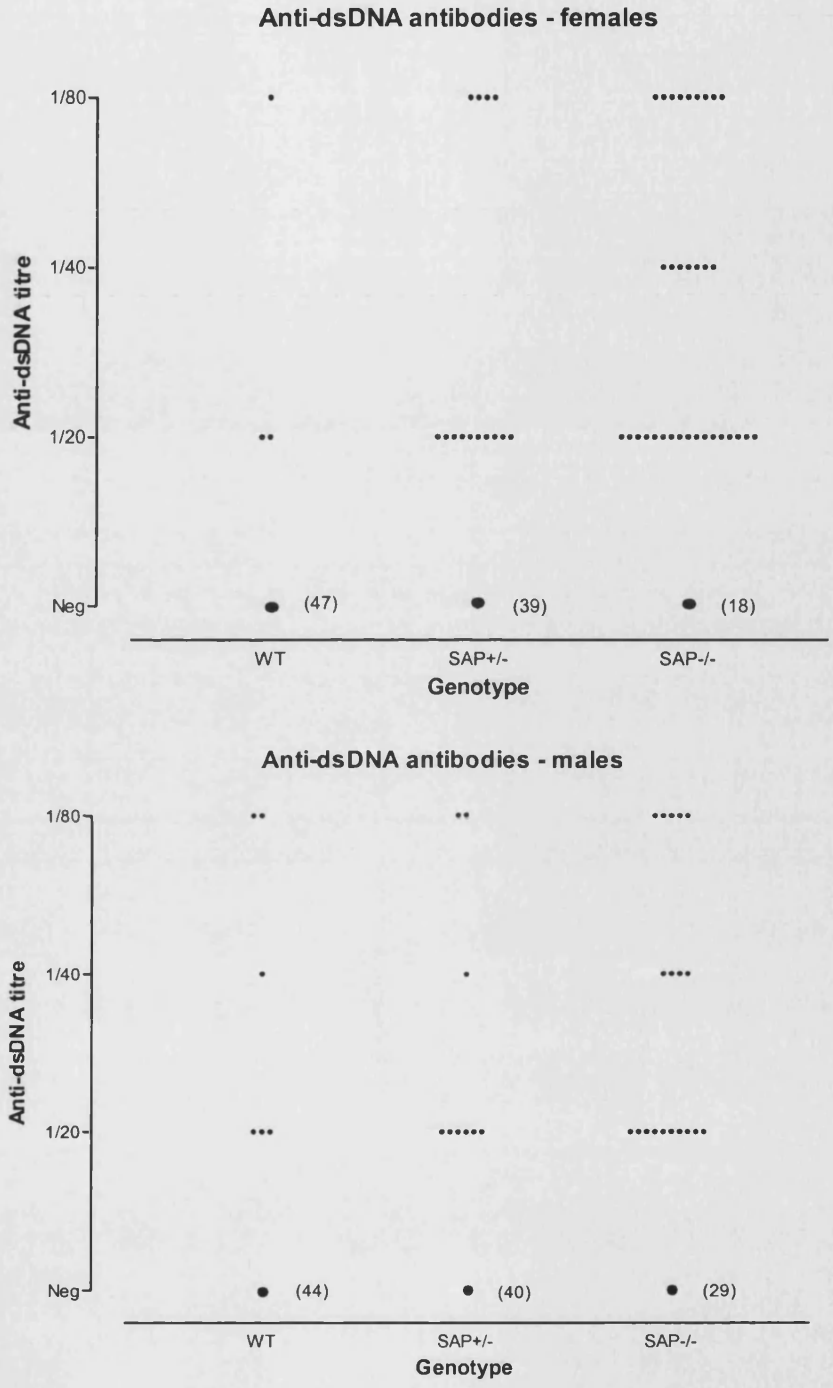

Figure 3.4 Anti-dsDNA antibody titres in female and male wild-type (WT) C57BL/6 mice compared to mice heterozygous for deletion of the SAP gene (SAP+/-) and mice homozygous for the SAP gene deletion (SAP-/-). There were significantly more female and male SAP-/- mice with anti-dsDNA antibodies compared to wild-type mice, with SAP+/- mice intermediate between them (Chi-squared test, P=0.003 for males and 0.001 for females).

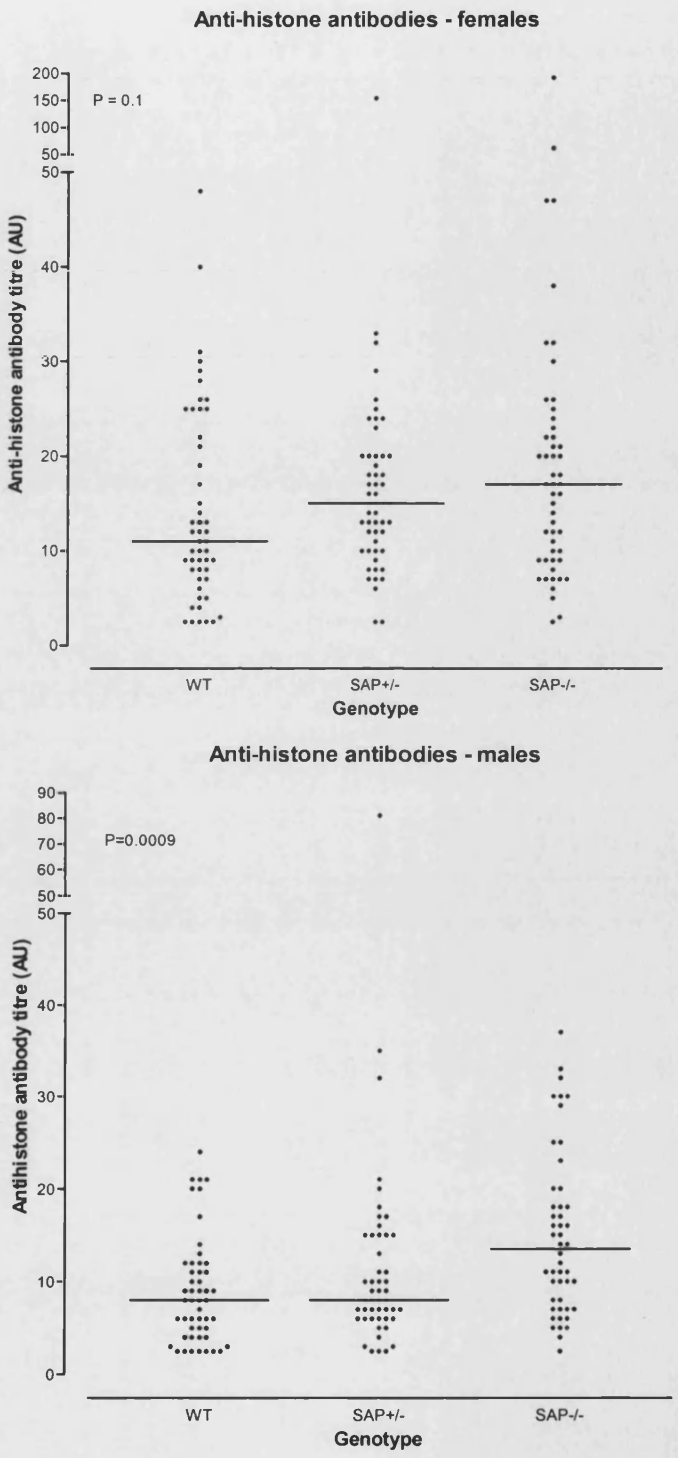

Figure 3.5 Anti-histone antibody titres at 12 months of age in wild-type (WT) C57BL/6 mice compared to mice heterozygous (SAP+/-) and mice homozygous (SAP-/-) for the SAP gene deletion. Significantly higher titres of anti-histone antibodies were seen in male SAP-/- mice compared to their wild-type (Mann-Whitney U test, P=0.0009) and SAP+/- (Mann-Whitney U test, P=0.01) counterparts. However, there was no significant difference in anti-histone antibody titres between female SAP-/- and wild-type mice (Mann-Whitney U test, P=0.1).

median = 36.5 units, range = 0-365) compared to SAP+/- mice (males: median = 5 units, range = 0-49; females: median = 12 units, range = 0-98) ($P < 0.0001$ for both sexes, Mann-Whitney U test), and in male and female SAP+/- mice compared to wild-type mice (males: median = 2.5 units, range = 0-49; females: median = 3 units, range = 0-240) (females, $P < 0.0001$; males, $P < 0.005$) (figure 3.6).

Significantly higher titres of anti-ssDNA antibodies were seen in male and female SAP-/- mice (males: median = 2.4 units, range = 0-22; females: median = 3.8 units, range = 0-26) compared to wild-type mice (males: median = 0 units, range = 0-7; females: median = 1.1 units, range = 0-9.8) (females, $P < 0.0001$; males, $P < 0.003$, Mann-Whitney U test). Interestingly, there were no significant differences in anti-ssDNA antibody titres between SAP-/- and SAP+/- mice of either sex (males, $P = 0.6$ and females, $P = 0.2$) (figure 3.7).

In order to analyse the relationship between the different autoantibodies detected, the presence and titres of antibodies from the cohort of SAP-/- mice were compared. The most significant correlation was between presence of ANAs and anti-chromatin antibodies (males: $r_{Sp} = 0.77$; females: $r_{Sp} = 0.85$).

In order to exclude the possibility of direct competition between SAP and autoantibodies for their respective nuclear antigens as an artefactual cause of apparently increased autoantibody titres in SAP-/- mice, autoantibody titres were

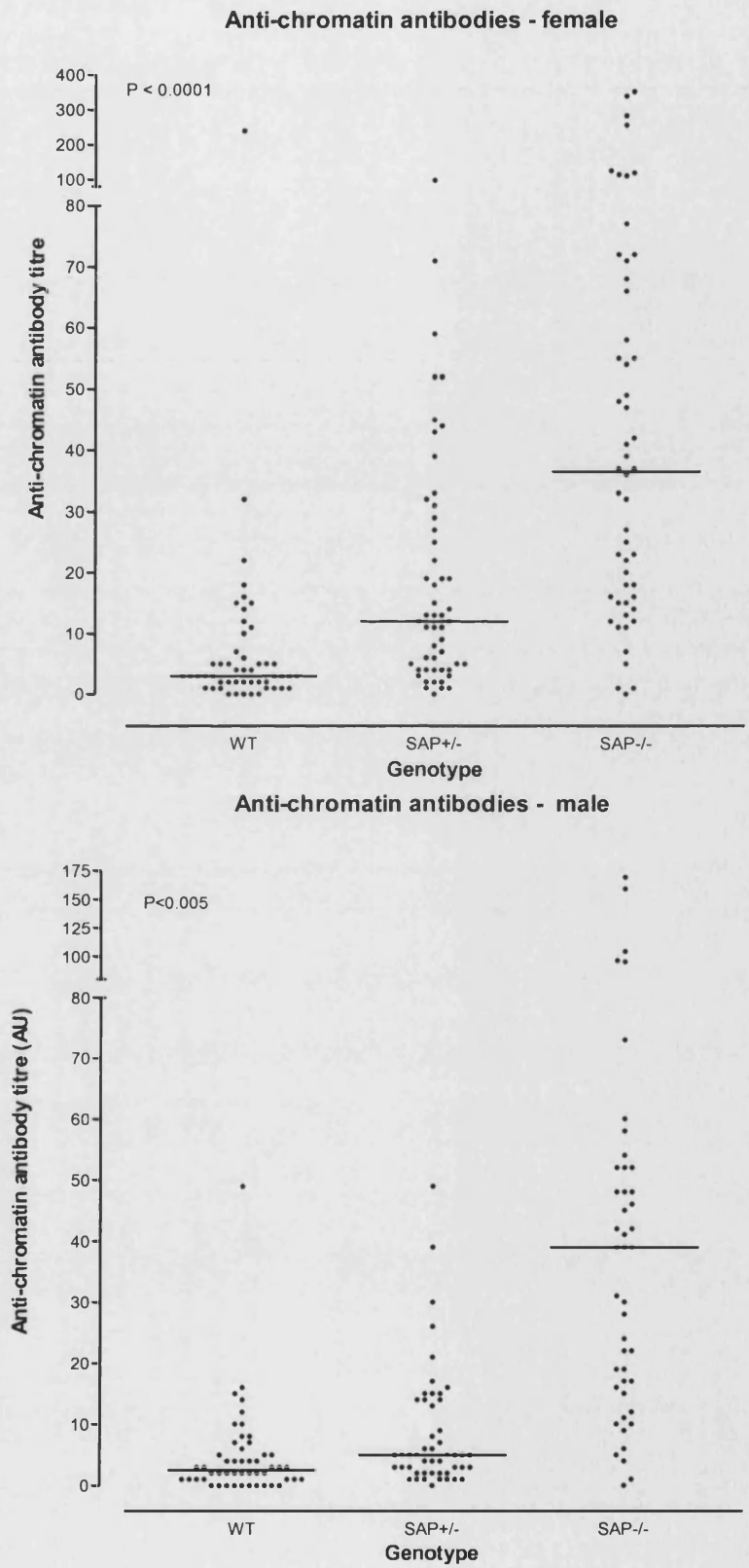

Figure 3.6 Anti-chromatin antibody titres at 12 months of age in wild-type (WT) C57BL/6 mice compared to SAP+/- and SAP-/- mice. Significantly higher titres were seen in both male and female SAP-/- mice compared to SAP+/- mice (Mann-Whitney U test, $P<0.0001$), and in SAP+/- mice compared to wild-type mice (females, $P<0.0001$; males, $P<0.005$).

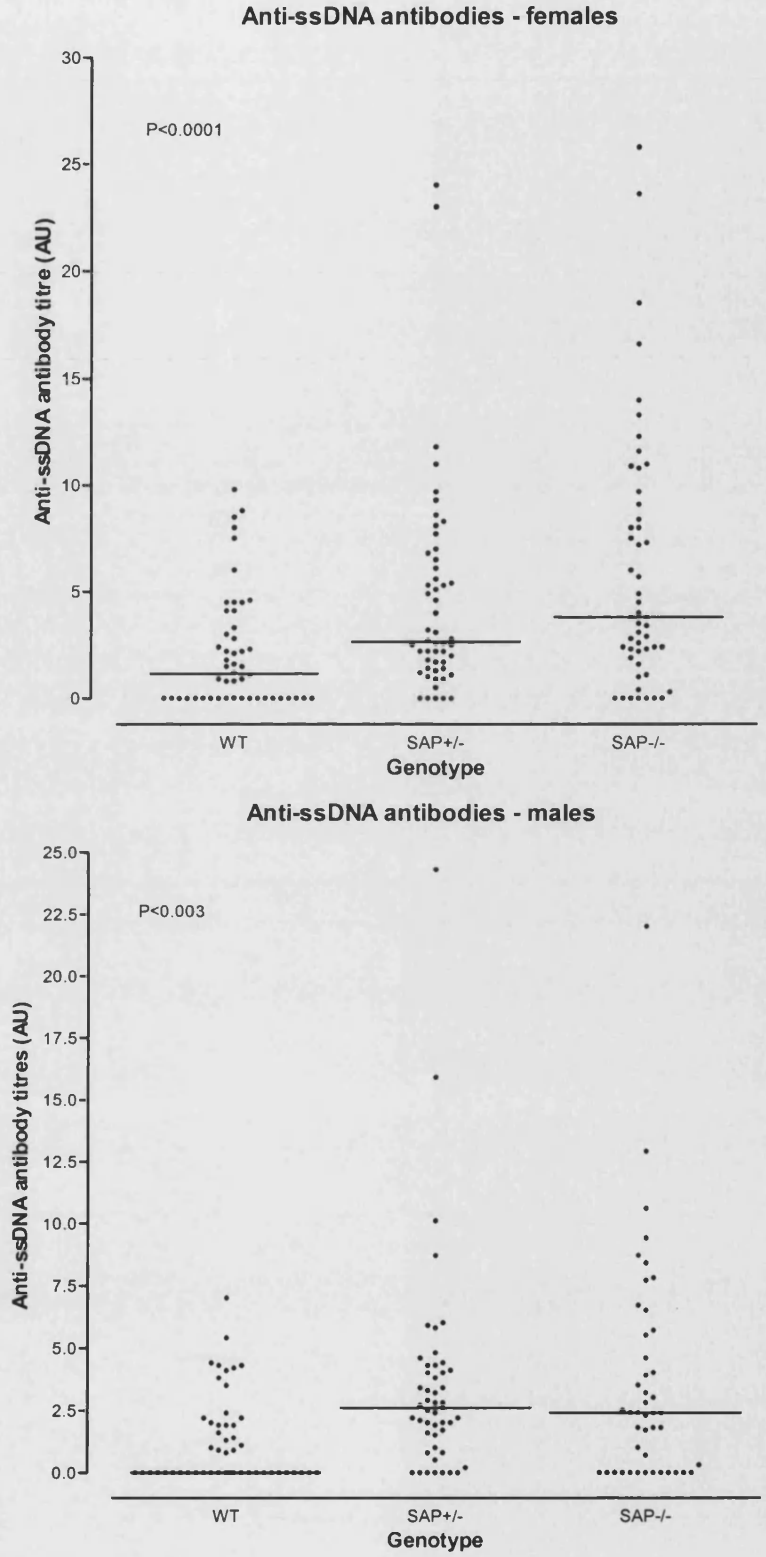

Figure 3.7 Anti-ssDNA antibody titres at 12 months of age in wild-type (WT) mice compared to SAP+/- and SAP-/- mice. Significantly higher titres were seen in both male and female SAP-/- mice compared to their wild-type counterparts (Mann-Whitney U test; females, $P<0.0001$ and males, $P<0.003$).

compared in SAP-/- serum before and after addition of pure mouse SAP at concentrations of 8.7 μg/ml and 87 μg/ml. There was no significant changes in anti-nuclear, anti-dsDNA, anti-histone, or anti-chromatin antibody titres following addition of mouse SAP (table 3.1), thus excluding direct competition between SAP and autoantibodies for antigen.

Urinary Analysis

At 12 months of age, urine was collected from each mouse over the 24 hours prior to exsanguination. There was no significant difference in creatinine clearance between wild-type mice (males: median = 0.10 mls/min, range = 0.03-0.18; females: median = 0.08 mls/min, range = 0.03-0.16), SAP+/- mice (males: median = 0.11 mls/min, range = 0.03-0.26; females: median = 0.10 mls/min, range = 0.04-0.17) or SAP-/- mice (males: median = 0.10 mls/min, range = 0.02-0.21; females: median = 0.09 mls/min, range = 0.04-0.14) of either sex (P = 0.1, Mann-Whitney U test) (figure 3.8).

Albuminuria was determined by radial immunodiffusion, and was not significantly different between WT mice, SAP+/- mice and SAP-/- mice of either sex. The vast majority of mice had albuminuria of < 50 μg/ml (the lower limit of detection of the assay), which is considered normal for mice [336].

Autoantibody	Coded serum number	No mouse SAP	+ 8.7 μg/ml mouse SAP	+ 87 μg/ml mouse SAP	Change
Immunofluorescent assay		**Titre**	**Titre**	**Titre**	
ANA	160	1/2560	1/2560	1/2560	Nil
	165	1/320	1/320	1/320	Nil
Anti-dsDNA	160	1/80	1/80	1/80	Nil
	165	1/20	1/20	1/20	Nil
Immunoradiometric assay		**Counts per minute**	**Counts per minute**	**Counts per minute**	
Anti-histone	160	4418	4151	4040	< -10%
	165	1389	1259	1541	< +/-10%
Anti-chromatin	160	9440	9493	9595	< +2%
	165	1445	1409	1417	< -3%

Table 3.1 Comparison of autoantibody titres in serum from two SAP-/- mice before and after addition of 87 μg/ml or 8.7 μg/ml of pure mouse SAP. There was no significant change in antibody titres in the presence of SAP, thus excluding direct competition by SAP for antigen as an artefactual cause of apparently increased autoantibody titres in SAP deficient mice compared to wild-type animals.

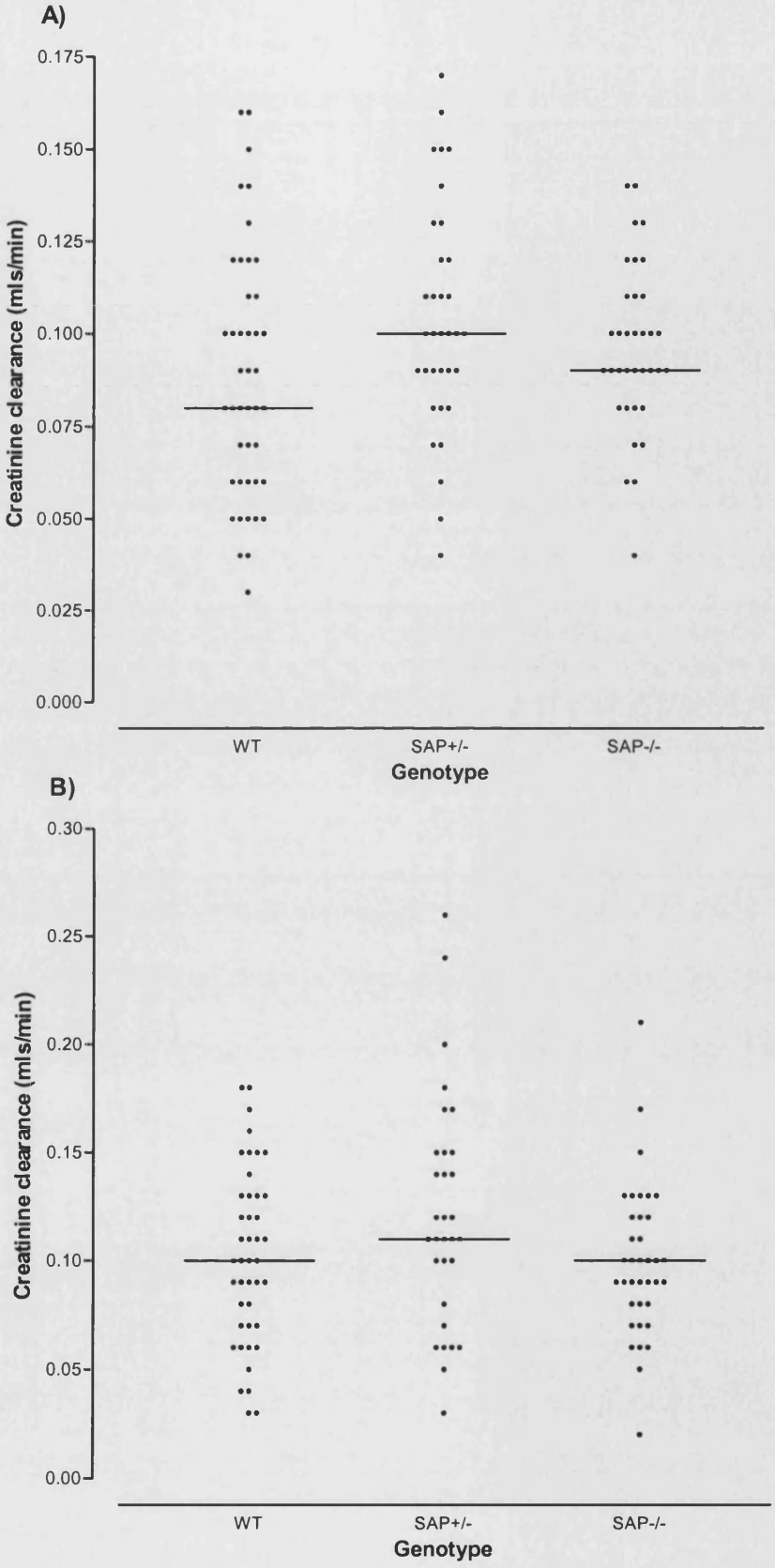

Figure 3.8 Creatinine clearance at 12 months of age according to genotype in female (A) and male (B) C57BL/6 mice. There was no significant difference between the creatinine clearance values in any of the genotypes in either sex.

Histology

In human SLE, anti-dsDNA and other antibodies are responsible for the immune complex vasculitis which underlies the pathology of the disease in the kidney and other organs.

Renal histology

Renal histology was examined in all mice. Coded sections of renal tissue were examined by Professor HT Cook, Department of Histopathology, Hammersmith Hospital, for the presence of glomerulonephritis as described in Materials and Methods.

At 12 months of age, glomerulonephritis was present in 75% of female SAP-/- mice, compared to 43% of female SAP+/- mice ($P < 0.0001$, Chi-squared test) and 16% of female wild-type mice (wild-type vs SAP+/- mice: $P < 0.0001$, Chi-squared test). Glomerulonephritis was less common and less severe in male mice, being present in 28% of SAP-/- mice, compared to 20% of SAP+/- mice and none of the wild-type SAP+/+ mice (wild-type vs SAP-/- mice: $P < 0.0001$ Fisher's exact test). Moderate or severe glomerulonephritis (grade II or III) was present in 38% of female SAP-/- mice compared to 9% of SAP+/- and 2% of wild-type females ($P < 0.0001$, Mann-Whitney U test), and in 6% of male SAP-/- mice, compared to none of the SAP+/- or SAP+/+ males (figure 3.9 and 3.10a).

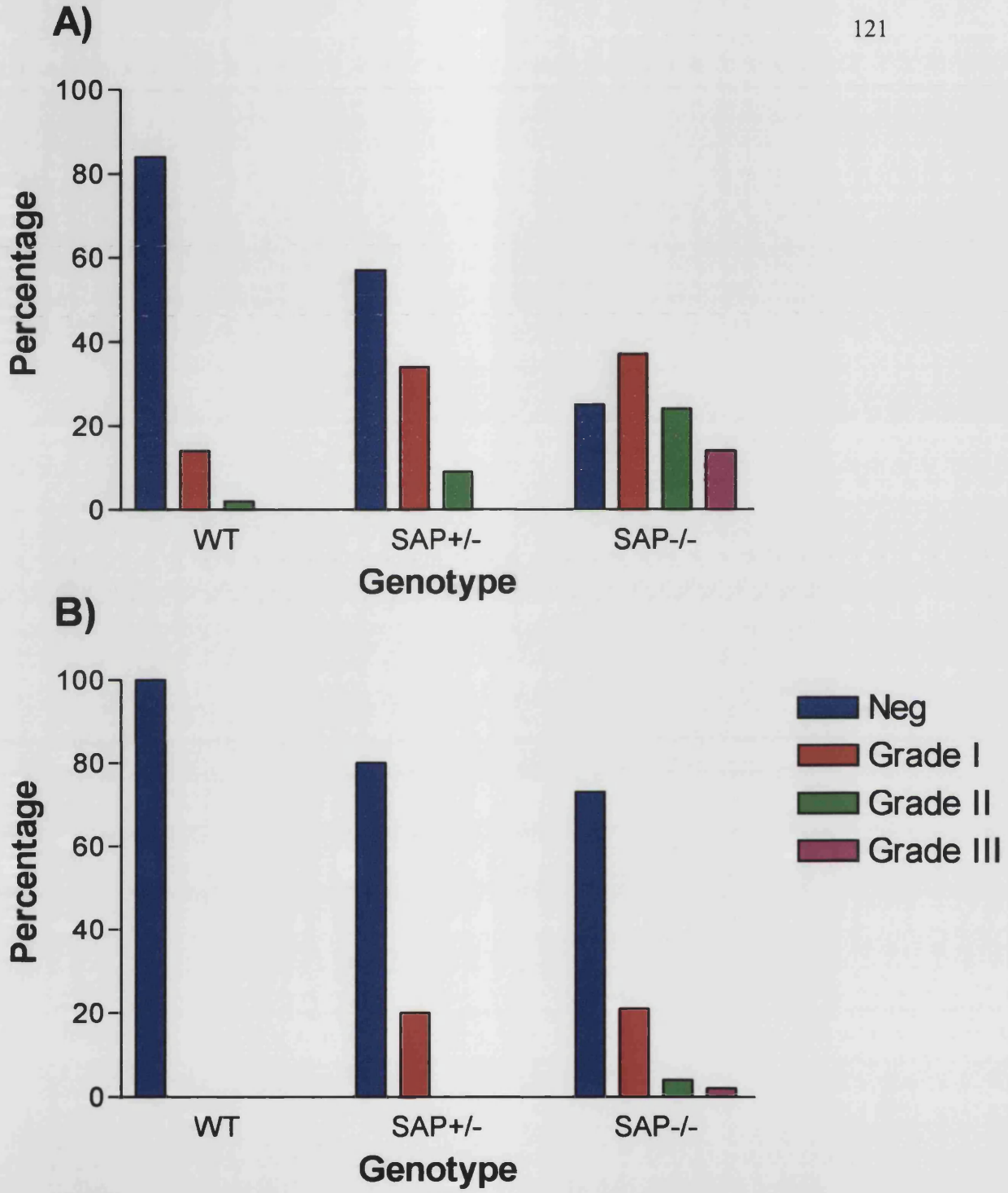

Figure 3.9 Glomerulonephritis at 12 months of age in female (A) and male (B) C57BL/6 mice. There was a highly significant increase in presence and severity of glomerulonephritis in SAP-/- mice compared to SAP+/- mice, and in SAP+/- mice compared to wild-type mice, with increased presence and severity of glomerulonephritis in females compared to males.

On univariate analysis, moderate/severe glomerulonephritis in female mice was associated with the presence ($P = 0.001$) and titre ($P < 0.0005$) of ANAs from 6 to 12 months of age. Moderate/severe glomerulonephritis in female mice was also significantly associated with presence of anti-dsDNA antibodies ($P = 0.001$) and higher titres of anti-chromatin ($P = 0.0001$, median 26.8 vs 4.7 units) and anti-histone antibodies ($P = 0.05$, median 15.6 vs 12.6 units). Similarly, among male mice, moderate/severe glomerulonephritis was significantly associated with presence ($P < 0.005$) and titre ($P < 0.01$) of ANAs from 6 to 12 months of age, presence of anti-dsDNA antibodies ($P = 0.005$), and higher titres of anti-chromatin antibodies ($P = 0.002$, median 16.1 vs 4.7 units) and anti-histone antibodies ($P = 0.004$, median 15.1 vs 8.7 units). In addition, among males there was an association between high anti-ssDNA antibody titre and moderate/severe glomerulonephritis ($P = 0.01$, median 2.7 vs 1.9 units). Interestingly, there was no significant correlation/association between creatinine clearance or albuminuria and the presence of moderate/severe glomerulonephritis histologically.

Using a multivariate logistic regression model to assess relationships between all variables and moderate/severe glomerulonephritis, SAP-/- and SAP+/- mice were 11.5 ($P = 0.0001$) and 4 ($P = 0.006$) times more likely respectively, to have glomerulonephritis than wild-type mice. Similarly, females were 12 times more likely to have moderate/severe glomerulonephritis than males ($P = 0.0001$).

Representative samples of mouse kidney with and without glomerulonephritis were chosen, and the presence of immune complexes in the glomeruli was sought by direct immunofluorescence using sheep anti-mouse total IgG and goat anti-mouse C3. There was deposition of both IgG and C3 in the glomeruli of all mice with glomerulonephritis on light microscopy, but interestingly, IgG and C3 were also present in the glomeruli of mice with normal light microscopy. Staining was completely abolished by prior absorption of each antibody with pure antigen, confirming the specificity of fluorescent staining (figure 3.10b).

Electron micrographs of selected mouse kidneys in which there was severe glomerulonephritis on light microscopy showed markedly increased mesangial matrix accompanied by immune complex deposition (figure 3.10c).

Extra-renal histology

Histological abnormalities were sought in coded sections of skin, heart, lungs, liver, aorta, stomach, small and large intestine and salivary gland in all mice by Professor HT Cook, Department of Histopathology, Hammersmith Hospital and coded sections of spleen were examined by Dr R Hasserjian, Department of Histopathology, Hammersmith Hospital as described in Materials and Methods.

The skin, heart, stomach, aorta and small and large intestines were all histologically normal in over 99% mice, regardless of SAP genotype. Lymphoid infiltrates were present in the livers of 19.3% male and 28.1% female mice, in the lungs of 4.1%

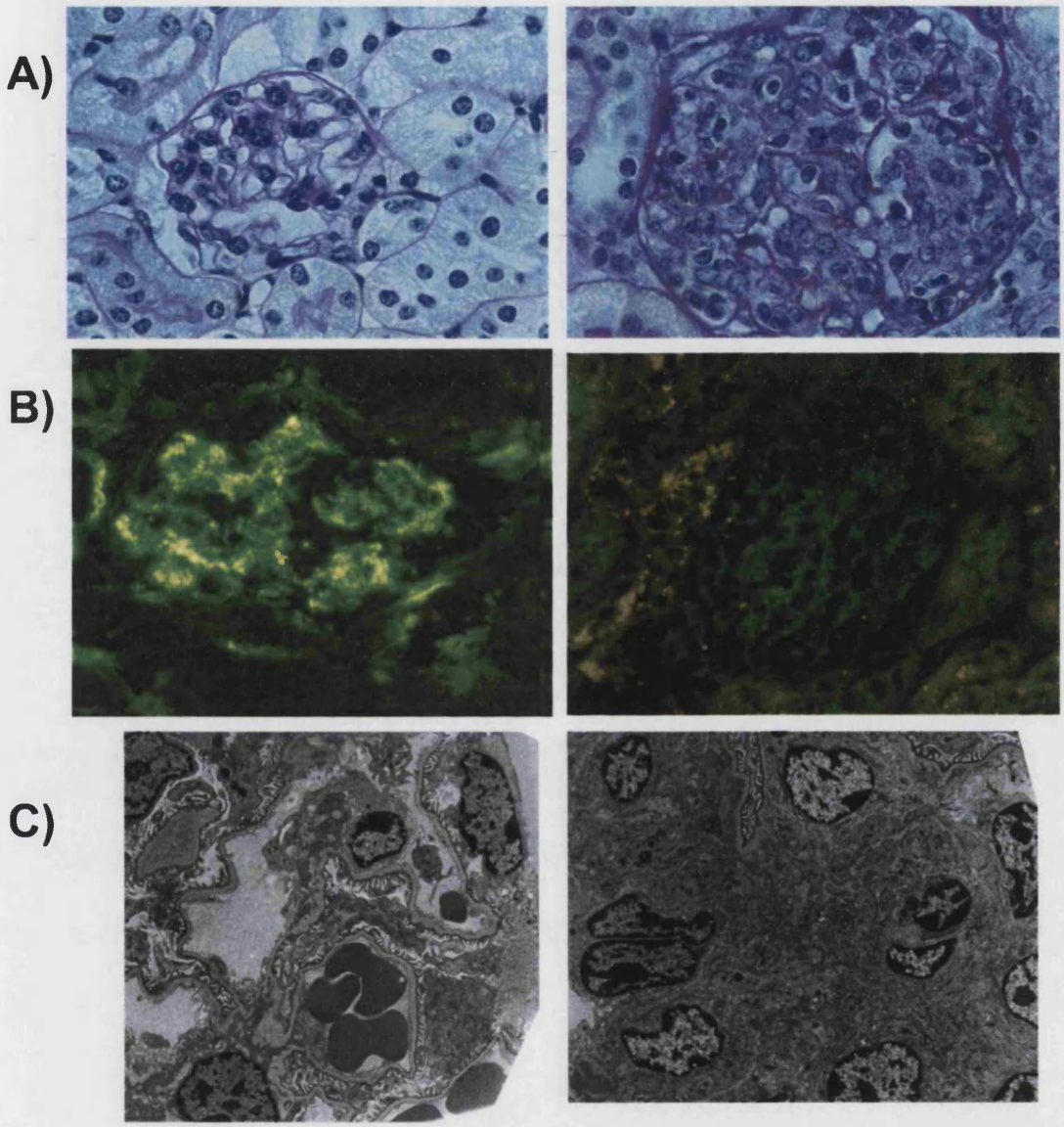

Figure 3.10 Glomerulonephritis in SAP-/- C57BL/6 mice.

A) Glomerulus with normal morphology (left) from a wild-type mouse. Haematoxylin and eosin staining, x250. Enlarged glomerulus from SAP-/- mouse (right), showing proliferative glomerulonephritis with increased mesangial matrix and marked hypercellularity with mononuclear cells in capillary loops. x250 B) Immunofluorescence staining with anti-mouse IgG (left) showing the granular appearance typical of immune complex deposition. Absence of immunofluorescence staining (right) following fluid-phase absorption of the fluorescent antibody conjugate, confirming specificity of staining. C) Electron micrograph of normal kidney (left) from a wild-type mouse. Electron micrograph of a kidney with proliferative glomerulonephritis (right) showing mesangial expansion and immune complex deposition.

males and 7.2% females, and in the salivary glands of 57.9% males and 66% females. However, there was no significant association between SAP genotype and abnormality of any of these organs.

Spleens were abnormal, as determined by "spleen lymphocyte score", in 62.5% (30/48) of SAP-/- female mice compared to 30.6% (15/49) of wild-type female mice ($P = 0.002$, Fisher's exact test) and in 48.9% of SAP-/- male mice compared to 20.4% of wild-type males ($P = 0.007$, Fisher's exact test). Spleens were abnormal in 58% and 38.8% of female and male SAP+/- mice respectively, not significantly different from SAP-/- mice. The spleen lymphocyte score is described in detail in Materials and Methods, but ranged from 0-9 (≥ 4 is abnormal) and was the sum of white pulp volume, white pulp coalescence, and red pulp lymphocytosis, each scored from 0-3.

The autoantibodies and histological abnormalities discovered in wild-type, SAP+/- and SAP-/- C57BL/6 mice at 12 months of age is summarised in table 3.2.

Comparison of autoimmunity in male and female mice

Several autoantibodies were more frequent and occurred at higher titre in female mice than in males, across all SAP genotypes. No autoantibody occurred significantly more frequently or in significantly higher titre in males compared to females.

		AUTOIMMUNITY						HISTOLOGY	
	N	Anti-nuclear		Anti-double stranded DNA	Anti-histone	Anti-chromatin	Anti-single stranded DNA	Glomerulonephritis	Splenic abnormality
		Number (%) +ve	Median titre (range)	Number (%) +ve	Median (range)	Median (range)	Median (range)	Number (%) +ve	Number (%) +ve
Female									
WT	50	20 (40)	0 (0-1/1280)	3 (6)	11.3 (2-48)	3.0 (0-240)	1.1(0-10)	8 (16)	15 (31)
SAP+/-	53	34 (64)	0 (0-1/1280)	14 (26)	15.1 (2-153)	11.9 (0-98)	2.7 (0-24)	23 (43)	29 (58)
SAP-/-	51	45 (88)	1/640 (0-1/2560)	33 (65)	16.7 (2-192)	36.5 (0-365)	3.8 (0-25.8)	38 (75)	30 (63)
		P=0.001	P=0.0001	P=0.001	P=0.1	P=0.0001	P=0.0001	P<0.0001	P=0.002*
Male									
WT	50	11 (22)	0 (0-1/640)	6 (12)	7.9 (2-24)	2.5 (0-49)	0 (0-7)	0 (0)	9 (20)
SAP+/-	49	24 (49)	0 (0-1/1280)	9 (18)	8.2 (2-81)	4.5 (0-49)	2.6 (0-24)	10 (20)	19 (39)
SAP-/-	48	47 (98)	1/640 (0-1/2560)	19 (40)	13.5 (2-36)	38.8 (0-169)	2.4 (0-22)	13 (28)	22 (49)
		P=0.001	P=0.001	P=0.003	P=0.009	P=0.0001	P=0.003	P=0.001*	P=0.007*

P values obtained by Chi-squared test, Fisher's exact test or Mann-whitney U test as appropriate. * - Significance of wild-type versus SAP-/- only

Table 3.2 Summary of autoimmunity and glomerulonephritis in SAP+/+, SAP+/- and SAP-/- C57BL/6 mice at 12 months of age

Among wild-type mice, there was a higher frequency ($P < 0.01$, Chi-squared test) and titre ($P = 0.04$, Mann-Whitney U test) of ANAs at 12 months of age in female mice than males, and significantly higher titres in females compared to males of anti-histone antibodies ($P = 0.007$, Mann-Whitney U test). However, there was no significant difference in frequency or titre of ANAs between male and female SAP+/- mice or male and female SAP-/- mice.

Significantly more female than male SAP-/- mice had anti-dsDNA antibodies (65% vs 40%, $P = 0.01$, Chi-squared), and titres of anti-ssDNA antibodies were higher in SAP-/- females than males ($P = 0.02$, Mann-Whitney U test). Similarly, titres of anti-histone ($P = 0.0001$) and anti-chromatin ($P < 0.003$) antibodies were higher in SAP+/- females than males.

Importantly, there was considerably more frequent ($P < 0.007$, Chi-squared or Fisher's exact test) and severe glomerulonephritis ($P < 0.04$, Mann-Whitney U test) among female mice than males, across all genotypes. Among SAP-/- mice, glomerulonephritis occurred in 75% females compared to 28% males, and was markedly more severe in females ($P < 0.0001$, Mann-Whitney U test). Among SAP+/- mice, glomerulonephritis was present in 43% of females, in whom it was more severe ($P < 0.002$), compared to 20% of males. No male wild-type mouse had glomerulonephritis, but it was present in 16% of wild-type females. These sex differences are reminiscent of the 9:1 female predominance in human SLE, which is characterised by ANAs and immune-complex glomerulonephritis.

The frequency of abnormal splenic histology was significantly higher in female SAP+/- mice (P = 0.01, Fisher's exact test), and female SAP-/- mice (P = 0.05) than males of the same genotype.

ABSENCE OF SPONTANEOUS AUTOIMMUNITY IN PURE-LINE 129/SV SAP DEFICIENT MICE

The deleted SAP gene was backcrossed for 6 generations onto the inbred 129/Sv mouse strain by Dr M Botto, Department of Rheumatology, Imperial College School of Medicine to produce "pure-line" 129/Sv SAP deficient mice. A cohort of 51 "knockout" SAP deficient (SAP-/-) mice, 27 males (52.9%) and 24 females (47.1%) were followed for 18 months, all housed under identical conditions.

During the course of follow up, 2 female mice were found dead at 14 and 17 months of age and 3 males were found dead at 14, 16 and 17 months of age, respectively. One female mouse was killed at 14 months due to an eye infection.

All available mice were bled from the tail vein at 6 and 12 months of age, and killed by bleed out from the axillary artery at 18 months, before harvesting the kidneys in each case.

Serological Analysis

Serum obtained at 6, 12 and 18 months of age was tested for the presence of anti-nuclear antibodies (ANAs). Samples were screened at a dilution of 1/80 and

titrated to end-point. At 6 months of age, ANAs were absent in all 129/Sv SAP deficient mice (0/51) and at 12 months of age ANAs were found at a titre of 1/80 in only 2 female SAP-/- mice and no males. At 18 months of age, ANAs were present in 6 of 21 (28.6%) female SAP-/- mice, at a titre of 1/80 in 3 cases and 1/160 in 3 cases, and in 7 of 26 (26.9%) males, at a titre of 1/80 in 4 cases, 1/160 in 2 and 1/320 in the remaining case. ANA staining was always homogeneous

The remaining serological analyses were carried out only on serum obtained at 18 months of age.

Anti-dsDNA antibodies were analysed as before, and samples were screened at a dilution of 1/20 and titrated to end point. Titres of 1/20 or above were considered positive. Antibodies to dsDNA were present in 1 of 21 (4.8%) female mice and 2 of 26 (7.7%) males, all at a titre of 1/20. Anti-chromatin and anti-ssDNA antibody assays were performed by immunoradiometric assay (IRMA) as before, and samples were considered positive when > 3 S.D. above the lower limit of detection. Antibodies against chromatin were present in 0 of 21 female mice (0%) and 2 of 26 (7.7%) males. Anti-ssDNA antibodies were present in 1 of 21 (4.8%) females and 3 of 21 (14.3%) male mice.

Histology

Renal histology was examined in all available mice. Coded sections of renal tissue were examined by Professor HT Cook, Department of Histopathology,

Hammersmith Hospital, for the presence of glomerulonephritis as described in Materials and Methods. At 18 months of age, glomerulonephritis by light microscopy was present in 26.3% of female SAP-/- mice, including two in which it was severe, and in 1 (4.3%) male mouse, in which it was moderate.

Attempted Induction of Anti-chromatin Autoimmunity in 129/Sv Mice

In order to test whether immunisation with exogenous chromatin would induce anti-nuclear autoimmunity in the 129/Sv mouse strain, and if so, whether this was enhanced by SAP deficiency, a cohort of 10-week old 129/Sv SAP-/- mice and age matched wild-type controls were immunised with avian chromatin.

Groups comprising 20 male and 16 female SAP-/- mice and 23 male and 18 female wild-type mice were given 100 μg per mouse of chicken erythrocyte chromatin suspended in complete Freund's adjuvant sub-cutaneously on day 0 and boosted with the same dose suspended in incomplete Freund's adjuvant on day +32. All mice were bled on day -1, +14, +28, +41 and +56.

All sera were screened for the presence of ANAs at a dilution of 1/80. No ANAs were detectable prior to chromatin administration and ANAs were not detected at any time point following chromatin administration in any mouse.

SPONTANEOUS AUTOIMMUNITY IN HUMAN SAP TRANSGENIC MICE

C57BL/6 mice, transgenic for the human SAP gene, were produced by Maeda's group in Japan [105], from whom they were obtained. These mice were crossed with our SAP-/- C57BL/6 mice by Dr M Botto, Department of Rheumatology, Imperial College School of Medicine, to produce mouse SAP-/-, human SAP transgenic pure-line C57BL/6 mice (SAP-/-, hSAP Tg). All offspring of SAP-/-, hSAP Tg mice were tested by PCR for the presence of the human SAP transgene by Dr DR Booth, and expression and concentration of human SAP were confirmed in individual experiments by rocket electroimmunoassay.

A cohort of 35 C57BL/6 SAP deficient mice, 16 males (45.7%) and 19 females (54.3%) were followed for 12 months, all housed under identical conditions. Thirteen (37.2%) of the mice were negative for the human SAP transgene (effectively SAP-/- mice), of which 7 were female and 6 were male; 22 (62.8%) were positive for the human SAP transgene (SAP-/-, hSAP Tg), of which 12 were female and 10 were male. Baseline concentration of human SAP was significantly higher in SAP-/-, hSAP Tg female mice (median = 93 μg/ml; range = 62-126) than in males (median = 23 μg/ml; range = 22-62) ($P = 0.0001$, Mann-Whitney U test).

At six months of age, there was no significant difference in presence or titre of ANAs in either sex, between SAP-/- and SAP-/-, hSAP Tg mice. Similarly, despite the expected increase in ANA titres with age in all mice, there was no difference at

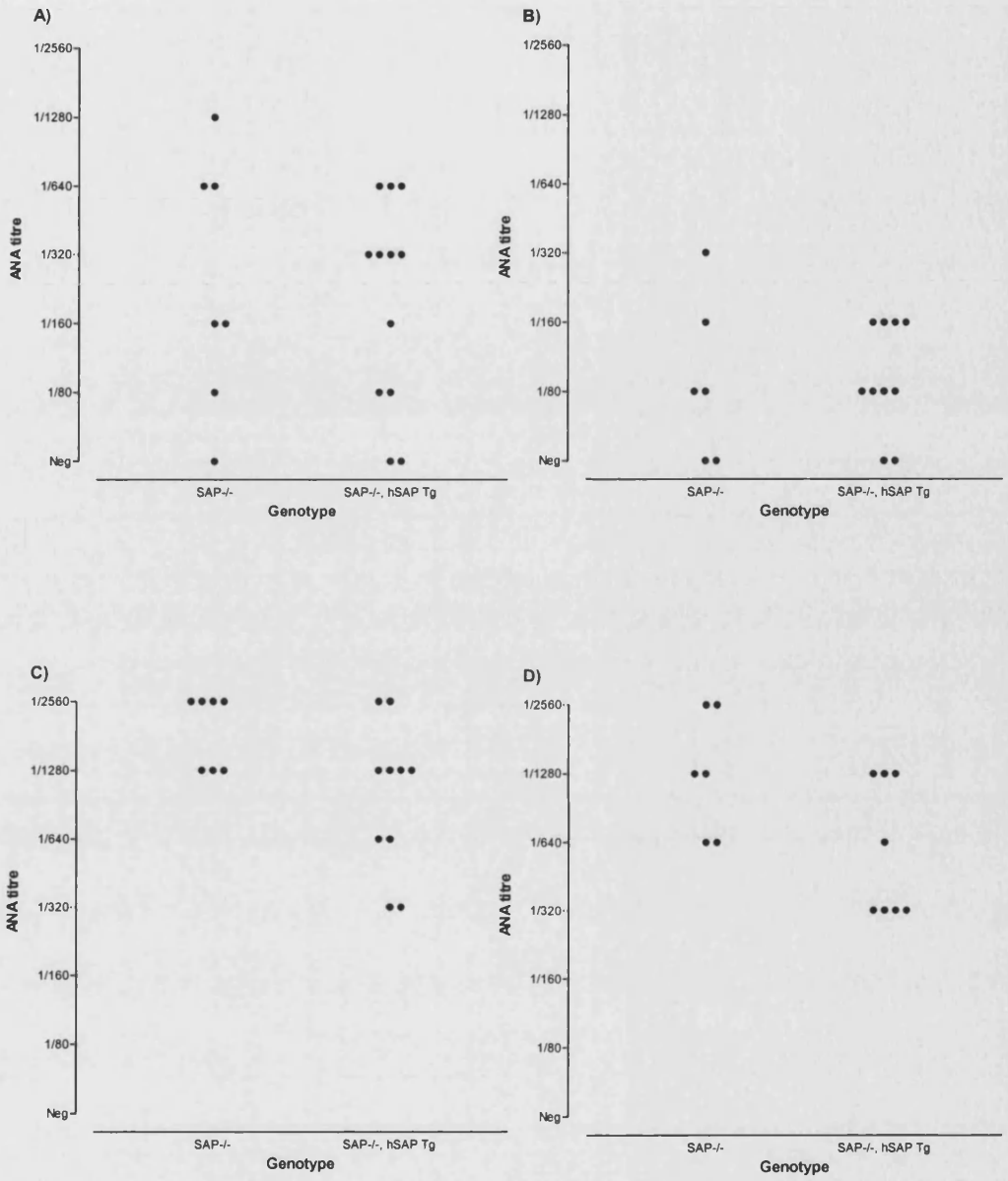

Figure 3.11 Comparison of spontaneous antinuclear autoimmunity between SAP deficient C57BL/6 mice, positive and negative for the human SAP transgene. Females are shown on the left (A, C) and males on the right (B, D). There was no significant difference in presence or titre of antinuclear antibodies at 6 months (A, B) or 12 months of age (C, D) between those mice expressing and those not expressing human SAP (Mann-Whitney U test, $P>0.05$ for all analyses). ANA titres were consistent with those previously shown in this chapter for SAP-/- mice (figure 3.3).

12 months of age between those mice expressing and those not expressing human SAP ($P > 0.05$ between all groups, Mann-Whitney U test) (figure 3.11).

The presence and titre of anti-dsDNA antibodies were not significantly different in either sex at 6 or 12 months of age, between those mice expressing human SAP and those without the human SAP transgene ($P > 0.05$ between all groups, Mann-Whitney U test) (figure 3.12).

The titre of anti-chromatin and anti-ssDNA antibodies was not significantly different at 6 months, between SAP-/- and SAP-/-, hSAP Tg mice of either sex ($P > 0.05$ between all groups, Mann-Whitney U test) (figure 3.13).

Renal histology was obtained at 12 months in all mice and coded slides were viewed by Professor HT Cook, Department of Histopathology, Hammersmith Hospital, for the presence of glomerulonephritis as described in Materials and Methods. There was no difference in frequency ($P > 0.2$, Fisher's exact test) or severity ($P > 0.2$, Mann-Whitney U test) of glomerulonephritis between SAP-/- and SAP-/-, hSAP Tg mice of either sex (figure 3.14).

Since the number of mice in this experiment were relatively small, analysis was also performed after grouping males and females together. At 6 months of age, there was no difference in frequency or titre of ANAs, anti-dsDNA, anti-chromatin or anti-ssDNA antibodies between SAP-/- and SAP-/-, hSAP Tg mice. At 12 months

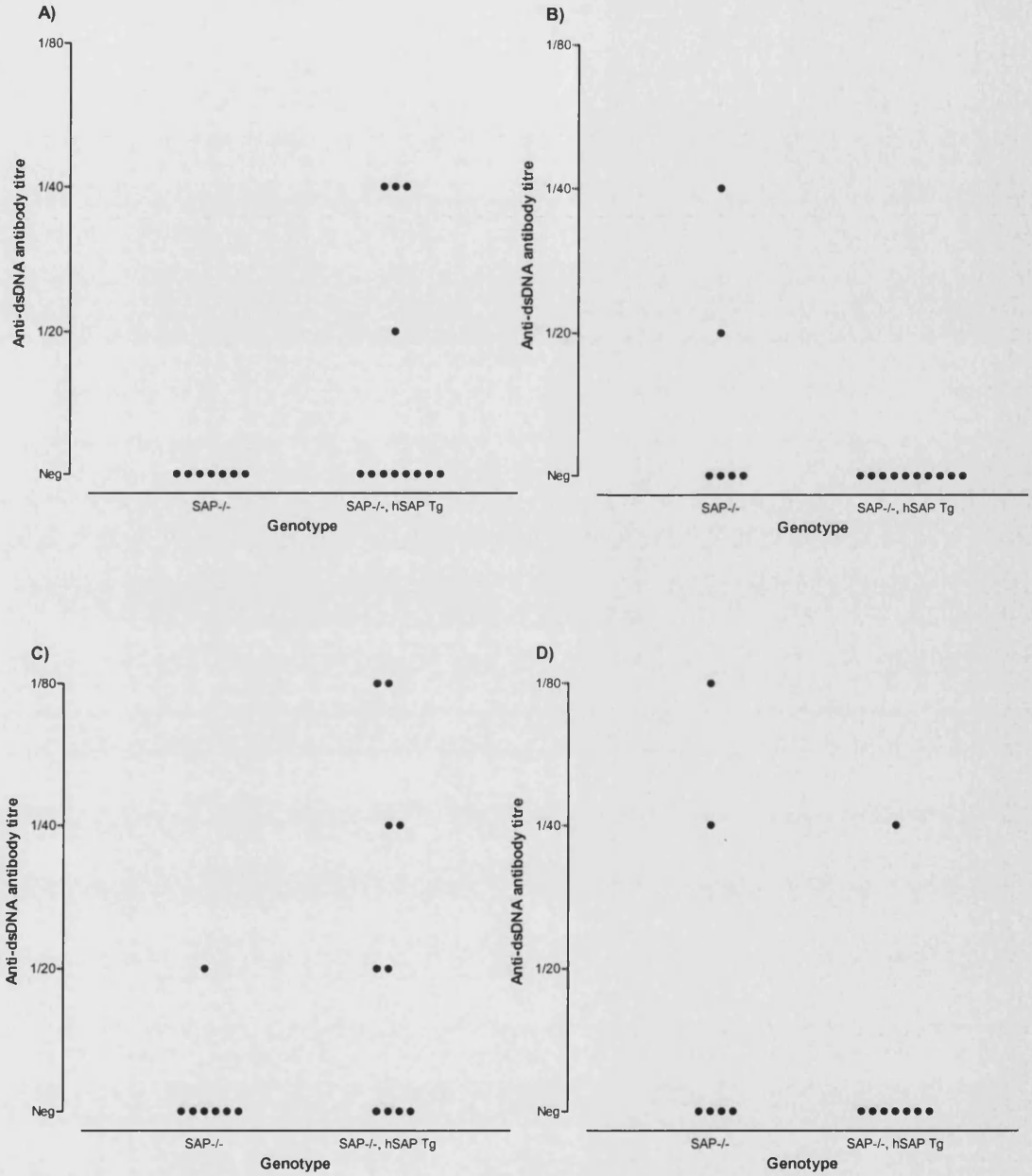

Figure 3.12 Comparison of anti-dsDNA antibodies between SAP deficient mice , positive and negative for the human SAP transgene. Females are shown on the left (A, C) and males on the right (B, D). There was no significant difference in presence or titre of anti-dsDNA antibodies at 6 months (A, B) or 12 months of age (C, D) between those mice expressing and those not expressing human SAP (Mann-Whitney U test, $P>0.05$).

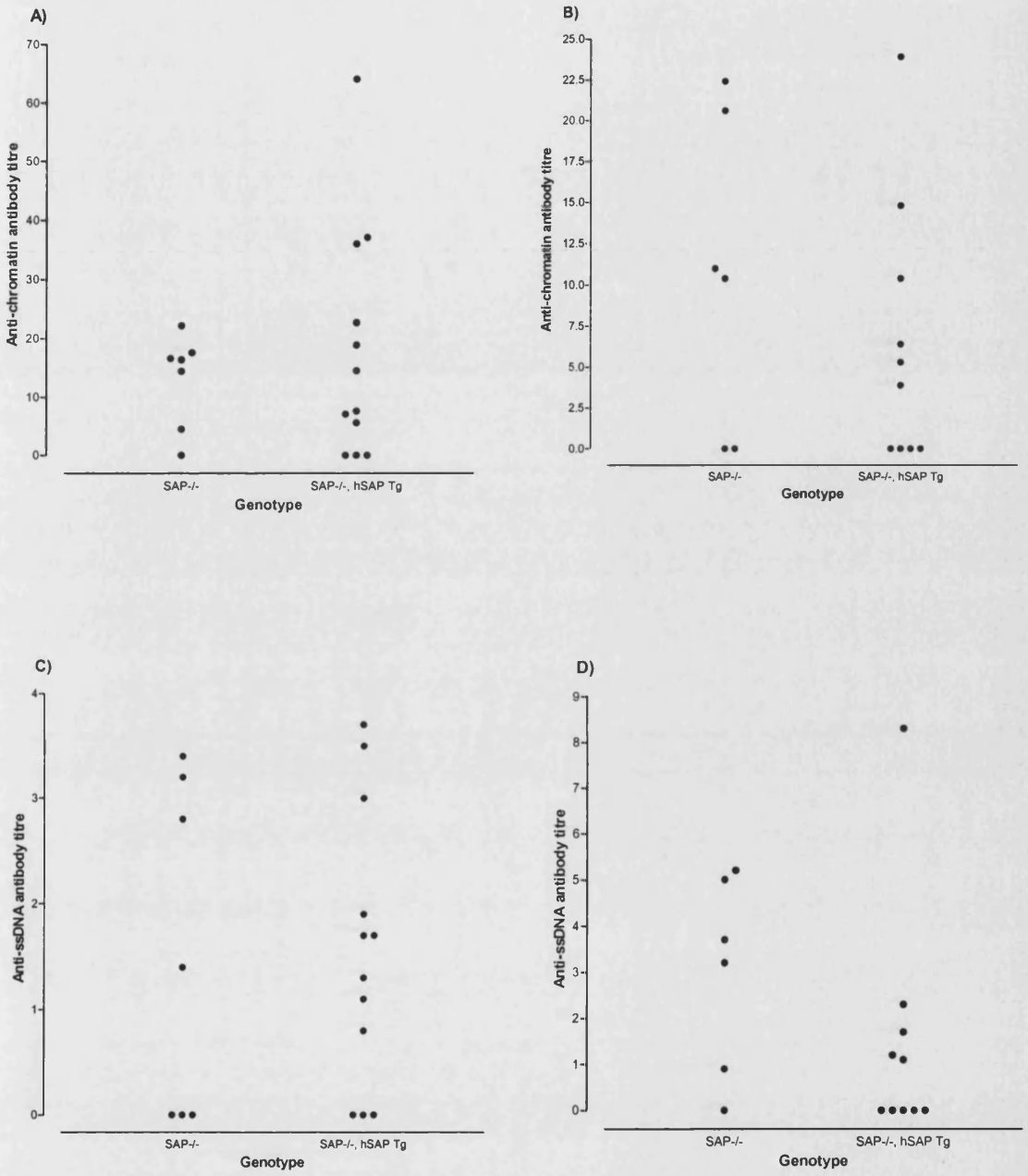

Figure 3.13 Comparison of anti-chromatin (A, B) and anti-ssDNA (C, D) antibody titres at 6 months of age between SAP-/- and SAP-/-, hSAP Tg mice. Females are on the left (A, C) and males are on the right (B, D). There was no significant difference in antibody titres between the genotypes (Mann-Whitney U test, $P>0.05$).

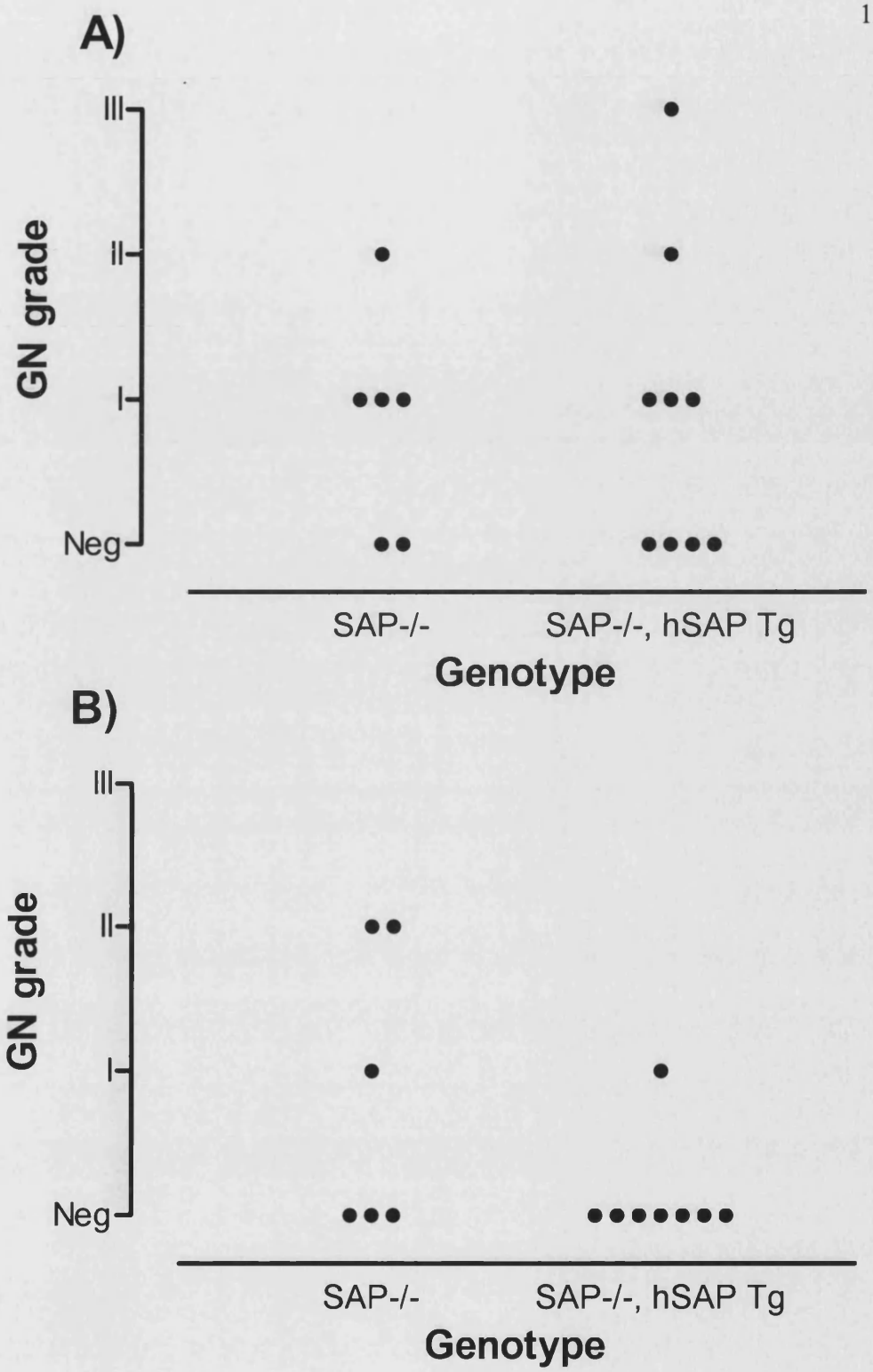

Figure 3.14 Comparison of presence and severity of glomerulonephritis between SAP-/- and SAP-/-, hSAP Tg females (A) and males (B) at 12 months of age. There was no difference between the genotypes (Fisher's exact and Mann-Whitney U tests, $P>0.05$).

of age, there was no difference in frequency or titre of anti-dsDNA antibodies, no difference in frequency of ANAs, and no difference in frequency or severity of glomerulonephritis between SAP-/- and SAP-/-, hSAP Tg mice. However, there were significantly lower ANA titres at 12 months of age in SAP-/-, hSAP Tg mice than SAP-/- mice ($P < 0.02$, Mann-Whitney U test).

Expression of human SAP in the C57BL/6 human SAP transgenic mice was noted by Maeda's group to reduce basal expression of native mouse SAP [105]. We confirmed this finding by electroimmunoassay for mouse and human SAP in a cohort of 23 pure-line C57BL/6 mice housed under identical conditions. Twelve of the 23 mice were shown to have the human SAP transgene, with a median human SAP concentration of 68 μg/ml (range: 31 to 140) and a median mouse SAP concentration of 0 μg/ml (range: 0-53). Among 11 littermate control mice in which the human SAP gene was absent, the median mouse SAP concentration was 13.5 μg/ml.

Since absence of mouse SAP in our C57BL/6 SAP deficient mice was associated with the autoimmune phenotype described earlier in this chapter, and since the phenotype was not abrogated by the expression of human SAP in these SAP deficient mice, we examined whether suppression of mouse SAP expression in human SAP transgenic C57BL/6 mice, might be sufficient to induce autoimmunity. The cohort of 23 mice were bled at 6 to 8 months of age with a view to bleeding out

Deletion of SAP gene
leads to highly significant
increase in spontaneous
anti-ds antibody production

↑ ANA

↑ GN

Female mice.

and obtaining renal histology at 12 months of age. All sera from the 6 to 8 month bleed were tested for the presence of ANAs.

ANAs were present in 1 of 7 female human SAP transgenic mice at 6 months of age and in 1 of 7 female mice in which the human SAP transgene was absent. There were no ANAs among 9 males, of which 5 expressed human SAP.

SUMMARY

Previous *in vitro* and *in vivo* studies had suggested that the pentraxins and, in particular SAP, may have a role in handling chromatin in order to prevent autoimmunisation. The present results suggest an important role for SAP in the handling of nuclear antigens *in vivo*. The inbred C57BL/6 mouse strain has a predisposition to development of autoimmunity; however, deletion of the mouse serum amyloid P component gene in this strain led to a highly significant increase in spontaneous autoantibody production with a global increase in anti-nuclear antibodies. The anti-nuclear autoimmunity was associated with development of immune complex glomerulonephritis, particularly in female mice, reminiscent of the 9:1 female predominance in the human equivalent, systemic lupus erythematosus. In addition, deletion of the SAP gene was associated with abnormal splenic histology with an increase in "lymphocyte score" as defined by red pulp lymphocytosis and white pulp volume and coalescence. Interestingly, the glomerulonephritis was clinically mild, despite often being histologically severe, and

was not associated with proteinuria or reduced creatinine clearance. SAP-/- mice survived normally to the age of 12 months.

However, the data presented here also highlights several important issues. Total SAP deficiency in the 129/Sv mouse strain was insufficient to induce spontaneous autoimmunity; indeed this could not be induced in 129/sv SAP deficient mice, even by administration of exogenous chromatin suspended in complete Freund's adjuvant. These data highlight the genetic complexity of autoimmunity, and in particular, systemic lupus erythematosus. Work in mouse models of human SLE have shown that susceptibility to development of the "full-blown" SLE phenotype is dependent upon complex genetic interactions involving several susceptibility loci.

A number of linkage studies have mapped genetic loci that influence the development and expression of SLE in both humans and mice. Genomic segments on murine chromosomes 1 (*Sle1)*, 4 (*Sle2)* and 7 (*Sle3)* are associated with disease susceptibility in multiple strain combinations, suggesting a strong influence on autoimmunity by genes within them, and the human syntenic equivalent of mouse *Sle1,* 1q21-44, has also been shown to confer strong disease susceptibility. Subsequent analyses of congenic mouse strains have provided a detailed characterisation of the component autoimmune phenotype produced by each of these susceptibility loci. *Sle1* in particular, but also *Sle3,* mediate the loss of immune tolerance to chromatin and lead to the appearance of high titres of antinuclear autoantibodies (ANAs) and initiation of autoimmunity. *Sle1* in isolation however,

does not cause the development of severe lupus nephritis, which appears to be mediated, in particular, by *Sle3* [216-218].

The SAP gene is located within the *Sle1* locus and the phenotype of our SAP deficient C57BL/6 mice was entirely consistent with the expected component SLE phenotype, with predominant loss of immune tolerance to chromatin and high titre ANAs, but relatively little severe lupus nephritis and normal survival to 12 months of age.

The DNA construct which resulted in deletion of the entire SAP gene was introduced into 129/Sv mouse embryonic stem (ES) cells, and cells lacking the SAP gene were injected into C57/BL6 blastocysts. By definition therefore, all our SAP deficient mice, including so-called "pure-line" C57BL/6 SAP-/- mice, were homozygous for the gene targeted 129/Sv allele on chromosome 1. One cannot exclude entirely the possibility, albeit remote, that the observed phenotype in our C57BL/6 mice was caused, not by deletion of the SAP gene that was targeted, but by an unknown 129/Sv gene on chromosome 1 linked to the SAP null allele. Another theoretical possibility would have been disruption of a nearby gene, as well as that encoding SAP during production of the DNA construct; however the complete SAP coding region that we selectively deleted definitely contained no other gene.

In order to exclude the possibility that the phenotype in our C57BL/6 SAP-/- mice was due to the interaction between a 129/Sv gene other than SAP and the remaining

C57BL/6 genome, we followed several lines of investigation. Firstly, we determined the spontaneous phenotype in pure-line 129/Sv SAP-/- mice. They did not develop significant autoimmunity by 12 months. However, the 129/Sv strain are non-predisposed to autoimmunity and thus its absence in SAP-/- mice does not preclude important involvement of the SAP gene. Secondly, we determined the spontaneous phenotype in C57BL/6 SAP-/- mice expressing human SAP. Human SAP did not abrogate the autoimmune phenotype seen in SAP deficient C57BL/6 mice. However, it would be surprising if human and mouse SAP functioned identically in the mouse, particularly given their different binding affinities *in vitro*, and therefore, the persistence of autoimmunity in human SAP transgenic mice lacking mouse SAP, does not exclude mouse SAP as the cause of the observed phenotype. Thirdly, we determined the spontaneous phenotype of human SAP transgenic C57BL/6 mice, in which mouse SAP expression is reduced. These mice did not have evidence of spontaneous autoimmunity at 6 months of age. However, the dynamics of human and mouse SAP expression is entirely unknown in this strain, and needs to be determined before any firm conclusions can be drawn.

We are currently in collaboration with Dr EK Wakeland, who has introduced extra copies of the mouse SAP gene into wild-type C57BL/6 mice. We plan to cross mice in which the extra SAP gene is expressed with our SAP-/- C57BL/6 mice and see whether the autoimmune phenotype is abrogated, which would definitively exclude any cause other than SAP deficiency for the autoimmunity.

Despite the remote possibility that the observed phenotype in SAP deficient mice is SAP-independent, we believe this to be unlikely; SAP is the major DNA and chromatin binding protein of the plasma, and its participation in the *in vivo* handling of such potentially autoantigenic epitopes, seems highly likely. The remainder of this work attempts to elucidate the mechanisms by which SAP may prevent autoimmunity.

Chapter 4 – Clearance And Degradation Of Chromatin And Nucleosomes

INTRODUCTION

There is strong evidence that the autoantibody response in systemic lupus erythematosus (SLE), the prototypic immune complex-mediated autoimmune disease, is driven by immunisation with self antigen [261-267]. The study of the clearance and processing of immunogens is therefore, central to understanding the pathogenesis of the disease.

Studies of clearance of autoantigens have focused primarily on the clearance of DNA from the circulation [337-341]. However, native DNA is poorly immunogenic [268] and evidence suggests that DNA is a target antigen rather than the primary immunogen in SLE. Autoreactive T cells from SLE patients recognise cationic DNA-binding proteins, and nucleosome-primed T cells, which predict the development of lupus nephritis, emerge spontaneously in lupus-prone mice before the serologic manifestations of autoimmunity. These findings suggested that DNA-protein complexes are the major immunogen in SLE [269-272], a hypothesis further strengthened by Datta *et al*, who found that immunisation of pre-autoimmune lupus mice with pure mononucleosomes accelerated development of severe lupus nephritis [273]. Burlingame *et al* demonstrated that early autoantibodies in lupus-prone mice recognised discontinuous epitopes on native chromatin and the

(H2A-H2B)-DNA subnucleosome and that as the immune response progressed, native DNA and other chromatin constituents generally became antigenic [274]. The same group compared the antigenicity of whole chromatin, DNA, denatured individual histones, and histone-histone and histone-DNA subnucleosome complexes and found that sera from patients with SLE reacted most frequently with whole chromatin, and that adsorption with chromatin removed the majority of reactivity with subnucleosome complexes and native DNA [275].

Studies of DNA in the circulation have revealed a size distribution consistent with the size of the nucleosome, and nucleosomes are the form in which DNA is released from cells dying by apoptosis [342]. There have been few reports concerning clearance or site of organ localisation of native long chromatin and nucleosome core particles from the circulation. Du Clos' group reported a half-life of 6.5 minutes for core particle clearance from the circulation [320]. Burlingame *et al* radiolabelled H1-stripped chromatin and nucleosome core particles with [^{125}I]Na, injected them intravenously and measured plasma clearance. In addition, they determined organ localisation 60 minutes following injection. However, tracer studies with proteins directly labelled by oxidative iodination are poorly informative about sites of clearance and catabolism because proteolysis is usually extremely rapid and the released iodotyrosine rapidly leaves the catabolising cell and is excreted in the urine. The amount of label associated with a particular tissue at any one time after injection, whether as catabolic products or intact protein, therefore, does not necessarily bear any relationship to the amount of protein catabolised by that tissue.

The "trapped catabolism" method depends on a common and straightforward principle. The subject protein is covalently linked to a tracer ligand, which is not itself degraded but is left behind in the catabolising cell upon degradation of the protein. The tracer ligand is therefore a cumulative marker of the amount of degradation that occurs in those cells. The method of choice for most applications of the trapped catabolism method is the tyramine cellobiose technique that was first described by Pittman *et al* [334]. Tyramine, the radioiodine acceptor, is linked to cellobiose, a non-hydrolysable reducing disaccharide, by reductive amination. The resulting tyramine-cellobiose (TC) adduct is then attached to the protein using cyanuric chloride. The method has been validated in several biological studies [334,343], but has not previously been used to determine catabolism of nucleosomes or chromatin.

Serum amyloid P component (SAP) is the major calcium-dependent DNA and chromatin binding protein of the plasma. SAP was shown in our laboratory to specifically displace H1 histone and solubilise native long chromatin [61]. In addition, SAP binds to apoptotic cells and it was suggested that its major physiological role might be to enhance non-immunogenic clearance of nuclear material, including chromatin, which is released from such cells. Our discovery of a spontaneous lupus-like phenotype in SAP deficient C57BL/6 mice with highly significant antinuclear autoimmunity, further suggested that the physiological role of SAP might be to enhance non-immunogenic clearance of nuclear material. Indeed, studies performed in our laboratory comparing whole body clearance of

radiolabelled chicken erythrocyte chromatin between [129/Sv x C57BL/6]F_2 SAP-/- and wild-type mice, revealed more rapid whole body clearance of radioactivity in female SAP deficient animals [232].

The studies presented in this chapter examine the handling of exogenously administered chromatin and nucleosomes by mice *in vivo,* and attempt, in particular, to determine the effect of SAP on their turnover. The rate of clearance of long chromatin was initially determined by whole body clearance of radioactivity; however, the technique was refined and subsequent studies compared clearance from the plasma of native long chromatin and nucleosomes in SAP-/- and wild-type C57BL/6 mice. Further studies using the "trapped catabolism method" determined the relative contribution by different organs to catabolism of nucleosomes, and specific cell types within the major catabolising organs were identified by dipping film autoradiography. Differences in the *in vivo* handling of chromatin and nucleosomes between SAP-/- and wild-type C57BL/6 mice were sought.

WHOLE BODY CLEARANCE OF LONG CHROMATIN

Following intra-peritoneal injection of directly-labelled long chromatin, there was no significant difference between wild-type, SAP-/- and SAP-/-, hSAP Tg mice in rates of whole body clearance of radioactivity (figure 4.1). This result contrasted data published from our laboratory, in which SAP-/- (129/Sv x C57BL/6)F_2 mice were found to clear long chromatin more rapidly than their wild-type counterparts [232]. In view of the possible inaccuracies associated with whole body clearance (due to

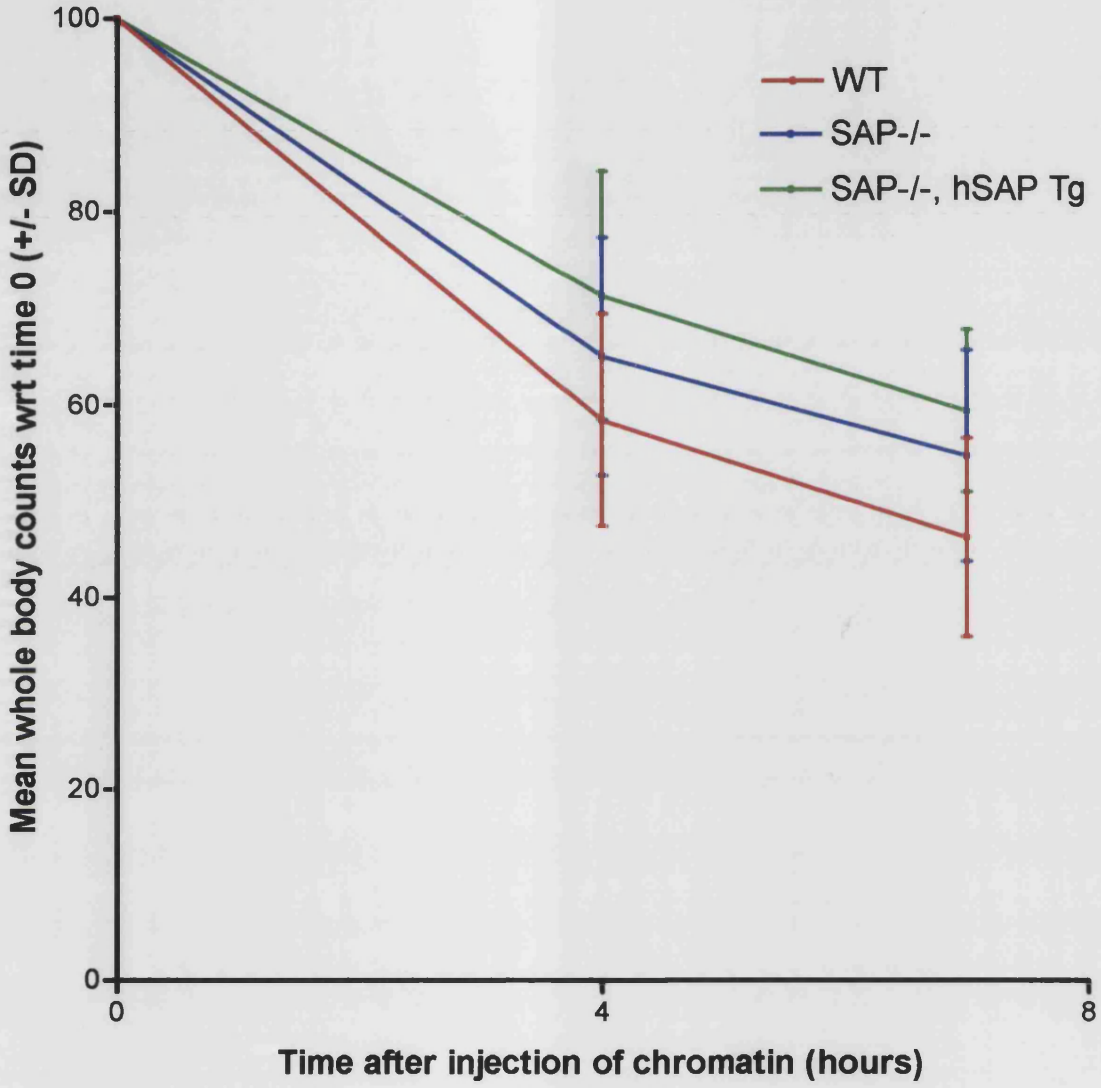

Figure 4.1 Whole body clearance of chromatin in wild-type C57Bl/6, SAP-/- C57BL/6 and SAP-/- C57BL/6 mice transgenic for human SAP (SAP-/-, hSAP Tg). There was no significant difference in the percentage of total chromatin cleared between the three genotypes after 4 hours.

bladder voiding and contamination of the fur with tracer) and our inconsistent results, all subsequent studies of clearance were designed to specifically examine clearance of chromatin and nucleosomes from the plasma after intra-venous administration, as described in Materials and Methods.

PLASMA CLEARANCE OF LONG CHROMATIN

Preliminary studies of plasma clearance of directly radioiodinated chromatin were performed in 4 wild-type C57BL/6 female mice and results are shown in figure 4.2.

In two separate experiments, clearance of long chromatin from the plasma was significantly more rapid in wild-type mice than in SAP-/- mice. In both experiments, at the 15 min time point, a significantly higher proportion of the injected chromatin had been cleared from the plasma in wild-type mice than SAP-/- mice, as shown in figure 4.3 ($P < 0.004$ and $P < 0.05$, Mann-Whitney U test). The rate of clearance of chromatin did not significantly differ between each experiment ($P > 0.5$, Mann-Whitney U test and Kruskal-Wallis test), and was therefore, independent of chromatin dose. For this reason, and since the experiments were identical in every respect other than chromatin dose injected, it was considered reasonable to pool the data from both experiments, which resulted in a highly significant difference between genotypes in the rate of plasma chromatin clearance at both 15 min ($P < 0.001$, Mann-Whitney U test and Kruskal-Wallis test) and 30 min ($P < 0.02$, Mann-Whitney U test and Kruskal-Wallis test).

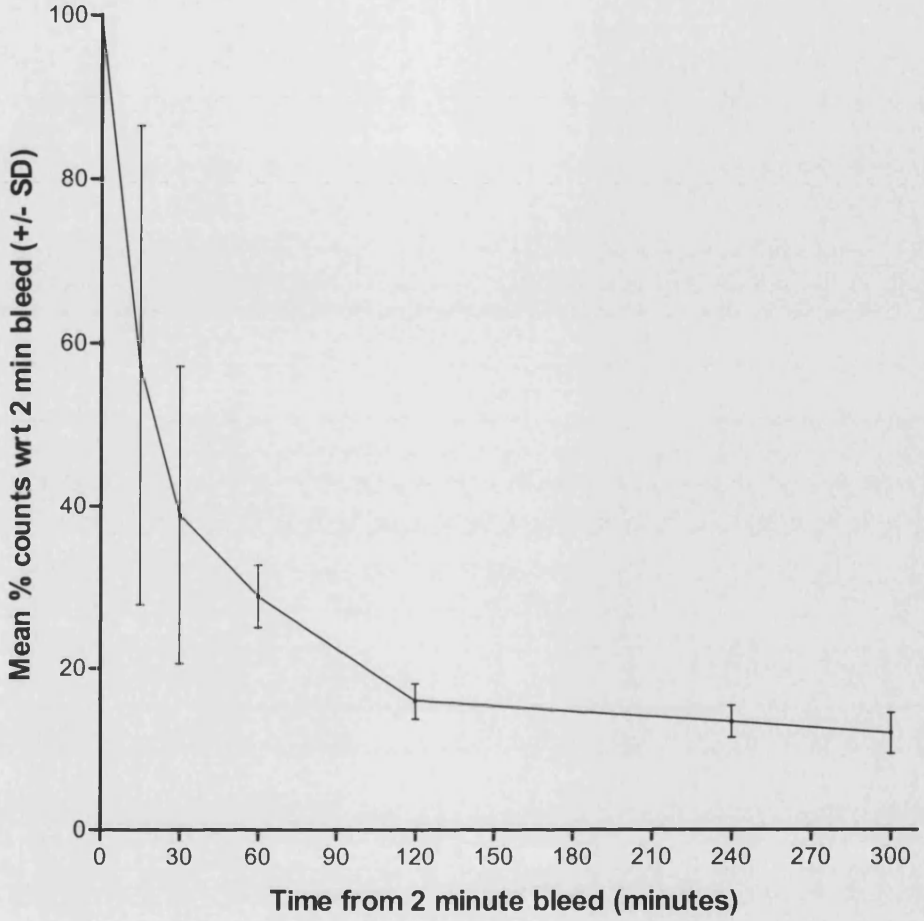

Figure 4.2 Clearance from the blood of long chicken erythrocyte chromatin in 4 wild-type C57BL/6 mice. Mean percentage counts at each timepoint represents the mean (+/-SD) counts per gram of blood from 4 animals expressed as a percentage of the counts per gram at 2 minutes after injection.

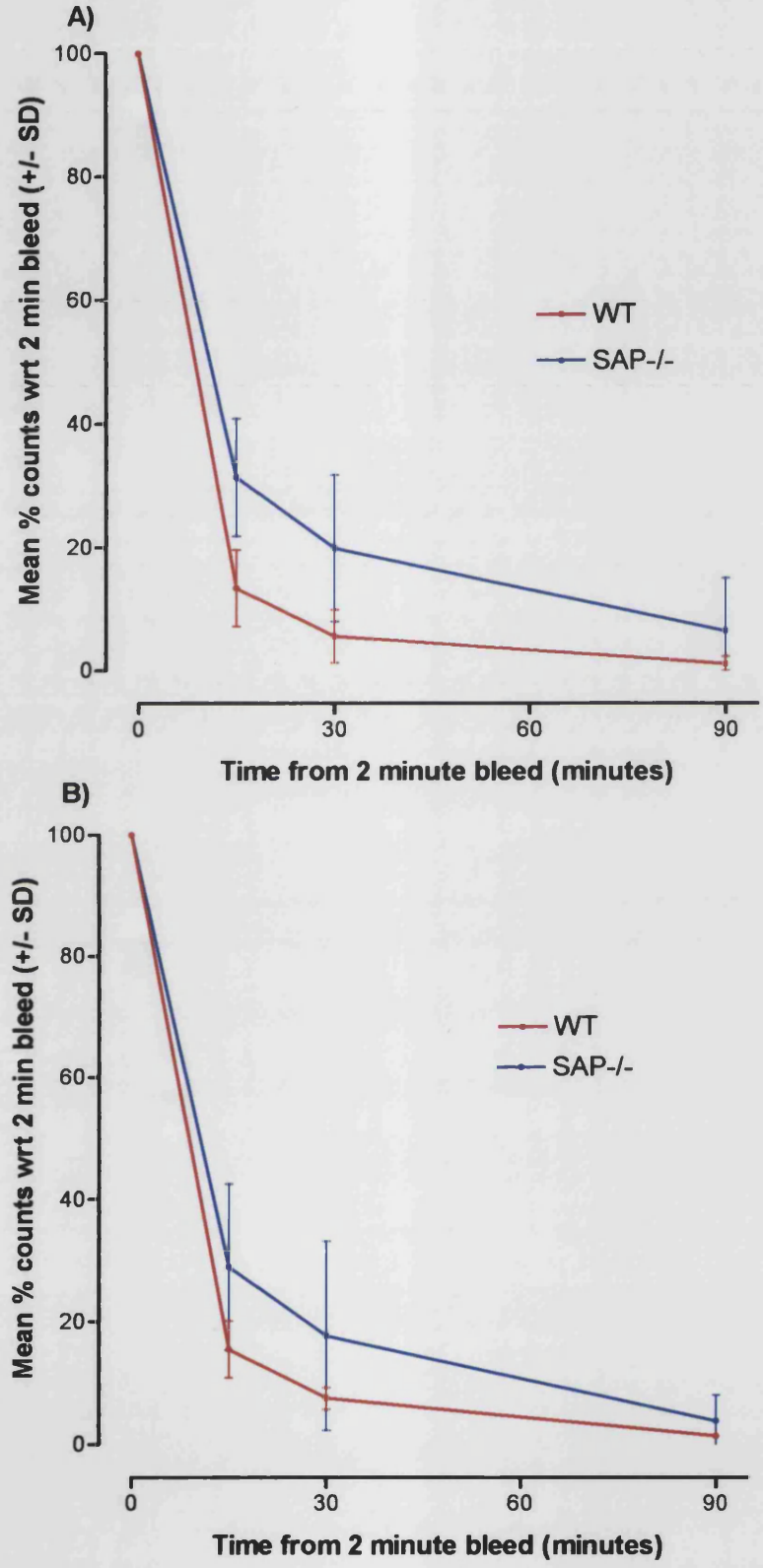

Figure 4.3 Clearance from the plasma of long chromatin from two separate experiments, A) and B). Both experiments show that at 15 minutes, wild-type mice had cleared a significantly higher proportion of the injected long chromatin compared to SAP-/- mice (At 15 minutes A; $P<0.004$ B; $P<0.05$, Mann Whitney U test).

PLASMA CLEARANCE OF NUCLEOSOMES

Nucleosomes were obtained from long chromatin. The size distribution of DNA fragments and the presence of all histone components (including H1 histone) in the final material were determined by DNA extraction followed by agarose gel electrophoresis and SDS-PAGE followed by staining with silver nitrate, respectively (figure 4.4). Together, these confirmed the predominance of intact mononucleosomes in the final material, which were directly radioiodinated as described in Materials and Methods. Following radioiodination, TCA precipitability of the labelled material was 91%.

Preliminary experiments were undertaken in order to roughly determine the rate of clearance of mononucleosomes from the blood, so that relevant time points could be selected for comparison of rates of clearance in SAP-/- and wild-type C57BL/6 mice. Results are shown in figure 4.5.

Plasma clearance of nucleosomes, shown in figure 4.6, was significantly faster in wild-type than SAP-/- C57BL/6 mice ($P < 0.04$ and $P < 0.03$ at 15 and 30 min respectively, Mann-Whitney U test).

In order to prove that the reduced rate of plasma clearance of nucleosomes observed in SAP-/- mice was caused specifically by SAP deficiency, we repeated the clearance experiments comparing 4 groups of mice. Using five weight-matched 6-8 week old female mice per group, the groups were as follows: wild-type, SAP-/-,

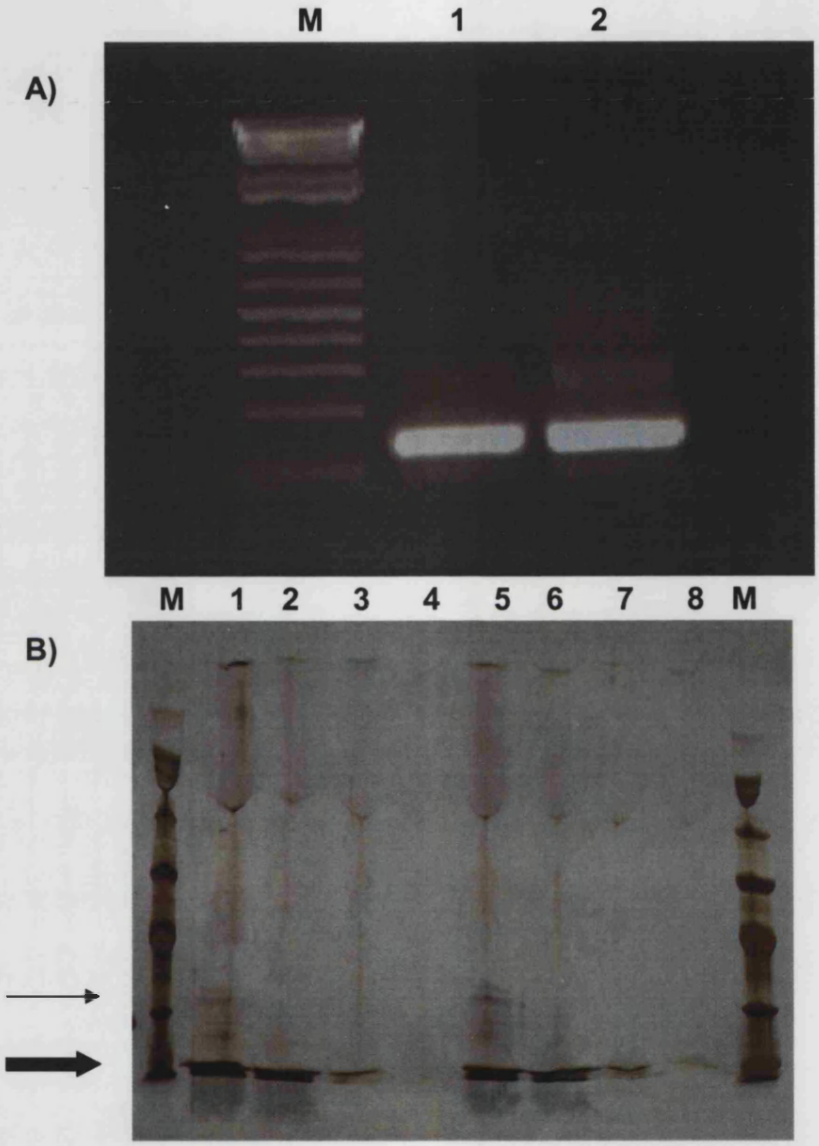

Figure 4.4 A) Agarose gel electrophoresis confirming the predominance of DNA fragments of between 100 and 200 base pairs in length, consistent with mononuceosomes. M is a 1 kilobase marker, lane 1 is chromatin that has been subjected to further digestion with micrococcal nuclease, and lane 2 is the calcium precipitated sample material. B) SDS-PAGE stained with silver nitrate confirming the presence of all histone components in the nucleosome preparation. Nucleosome core partricles (lanes 1-4) and long chromatin (lanes 5-8) were each loaded at 4 doses; 0.3 µg (lanes 4, 8), 0.9 µg (lanes 3, 7), 1.8 µg (lanes 2, 6) and 3.4 µg (lanes 1, 5). Histone H1 and H5 (thin arrow) were apparent in lanes 1 and 5, the four core histones (thick arrow) in lanes 3 and 7.

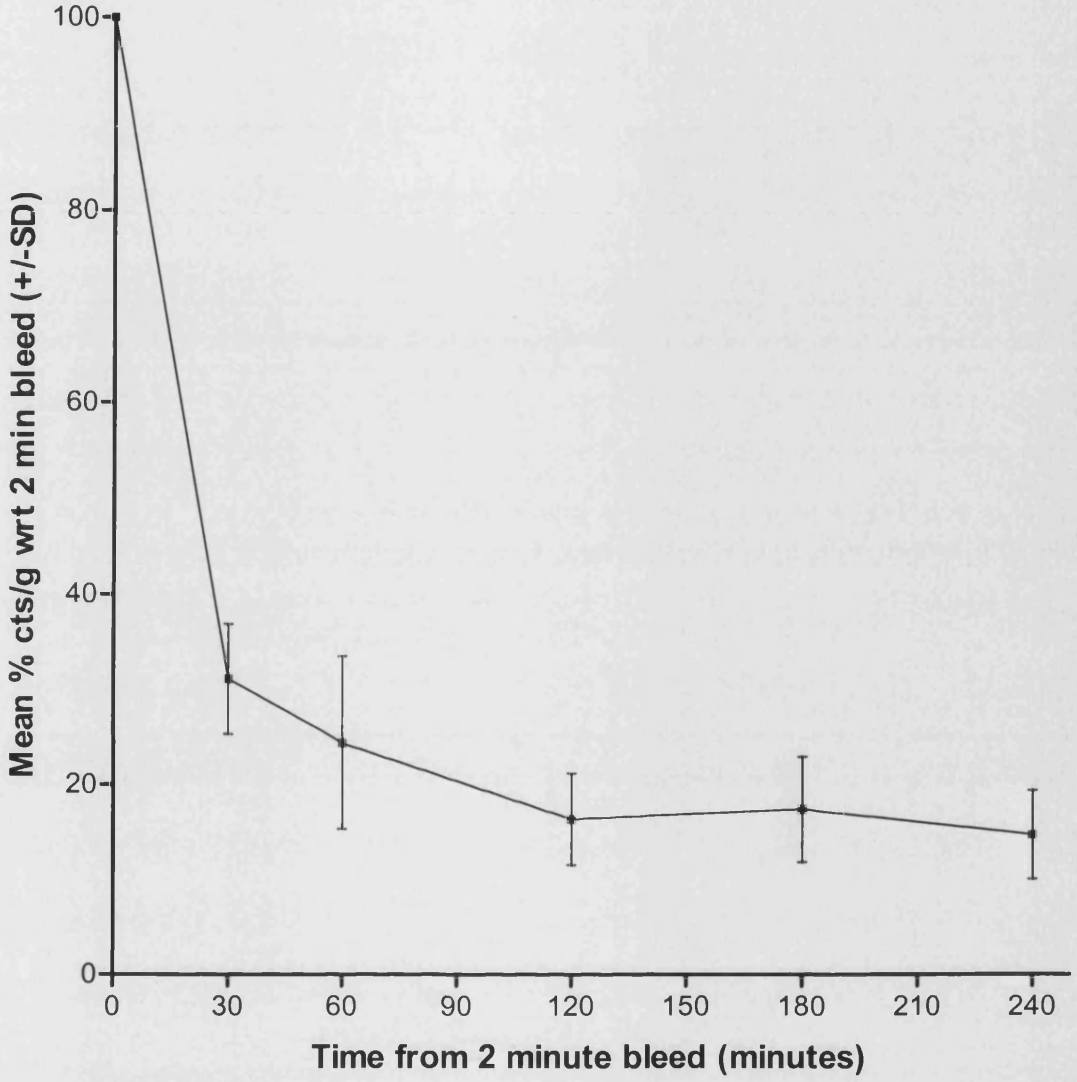

Figure 4.5 Clearance from the blood of mononucleosomes in 4 wild-type C57BL/6 mice. Mean percentage counts at each timepoint represents the mean (+/-SD) counts per gram of blood from 4 animals expressed as a percentage of the counts per gram 2 minutes after injection.

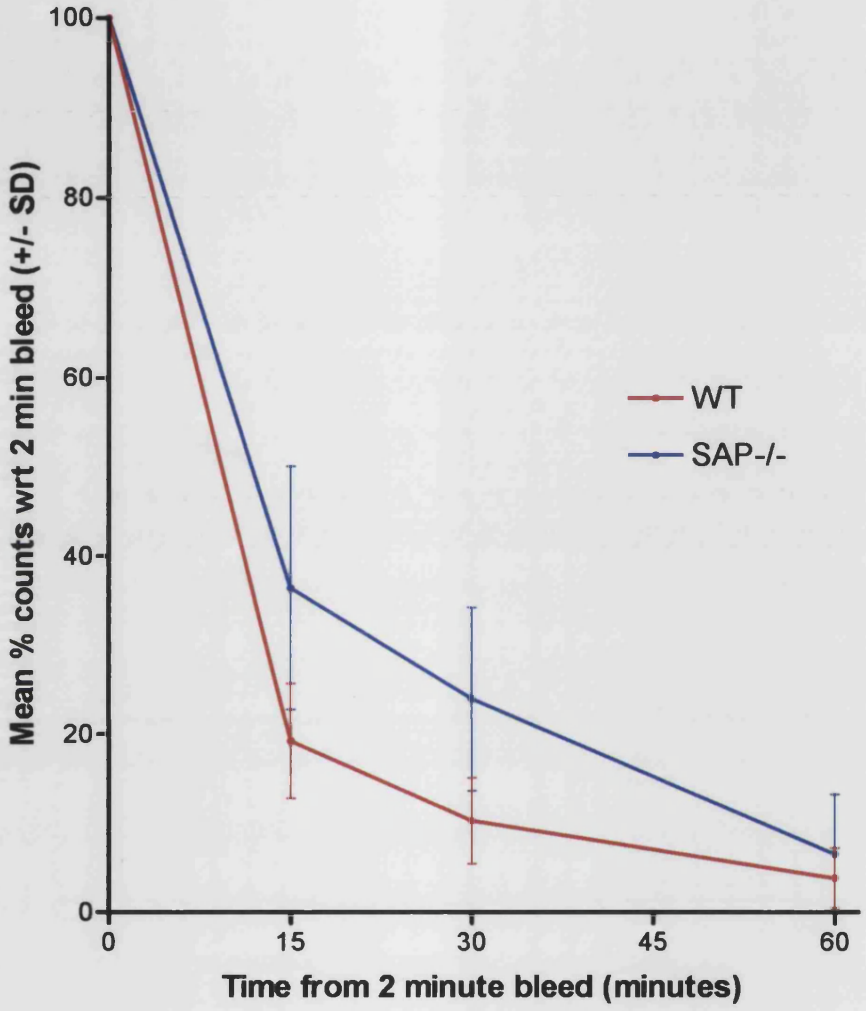

Figure 4.6 Comparison of plasma clearance of nucleosomes in SAP-/- and wild-type (WT) C57BL/6 mice. Clearance of nucleosomes from the plasma occurred significantly more rapidly in wild-type mice than in SAP-/- mice ($P<0.04$ and $P<0.03$ at 15 and 30 minute timepoints, respectively; Mann-Whitney U test).

SAP-/- after administration of mouse SAP, and SAP-/- after administration of human SAP.

Rates of nucleosome clearance from 2 consecutive experiments are shown in figure 4.7. In sharp contrast with previous results, rates of nucleosome clearance from the plasma in these experiments were not significantly different between wild-type and SAP-/- C57BL/6 mice. Interestingly, the nucleosomes injected in the latter two experiments were obtained by calcium precipitation of a different batch of long chromatin and subsequent DNA extraction and agarose gel electrophoresis of the chromatin revealed significant degradation of the starting material compared with that used as starting material for previous experiments (figure 4.8). Unfortunately, time did not permit preparation of a new batch of chicken erythrocyte chromatin for further investigation of plasma nucleosome turnover.

ORGAN LOCALISATION OF CATABOLISM OF NUCLEOSOMES

Confirmation of successful radioiodination of the tyramine cellobiose (TC)-labelled and native nucleosomes was obtained by autoradiography of the SDS-PAGE gel (figure 4.9).

In order for the "trapped catabolism" method to be considered biologically applicable, the tyramine-cellobiose (TC) labelled tracer must be metabolically indistinguishable from the tracer protein, at least through the step of irreversible

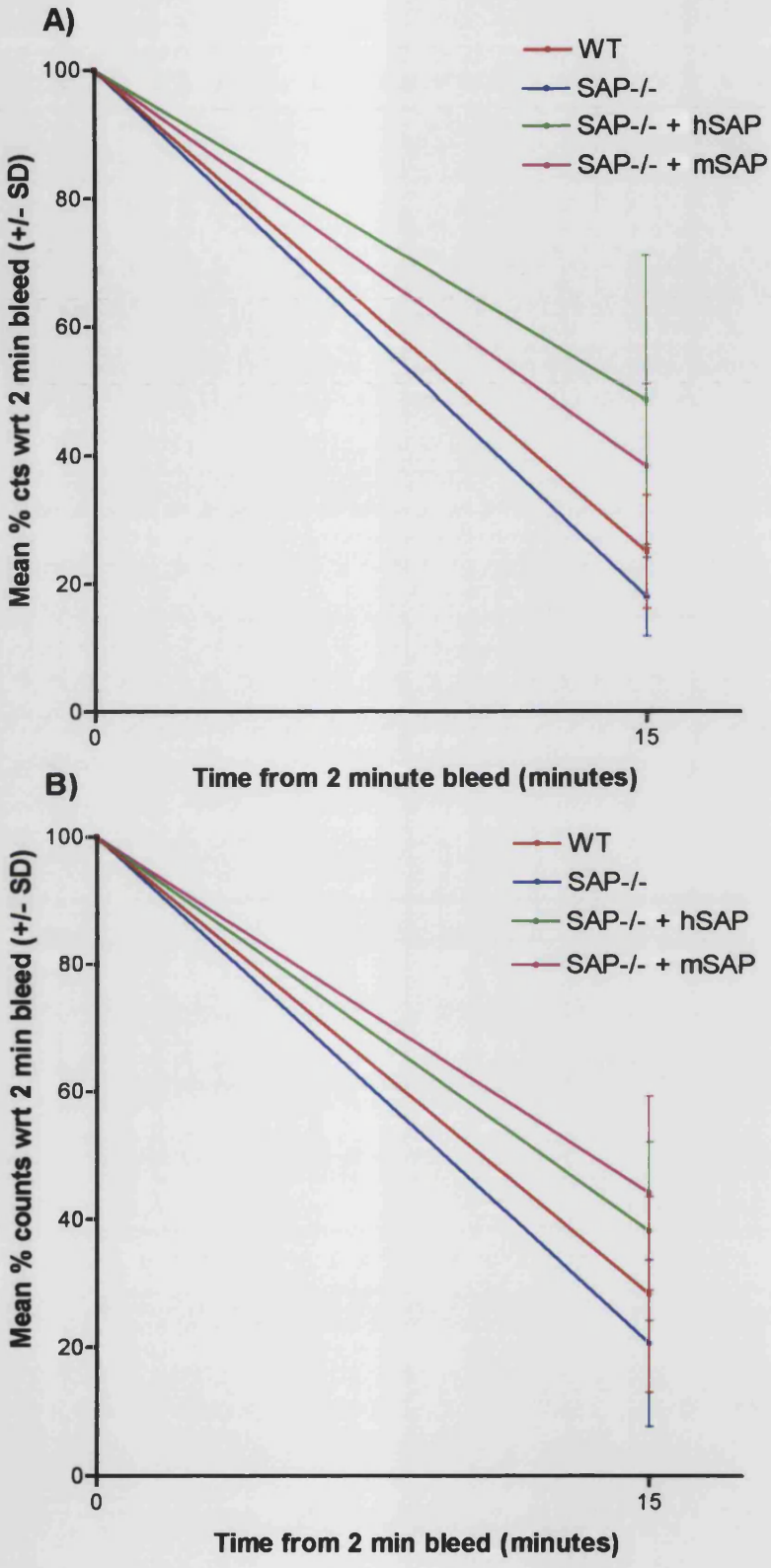

Figure 4.7 Two separate experiments, A) and B), showing rates of plasma clearance of mononucleosomes in 4 groups of C57BL/6 mice; wild-type, SAP-/-, SAP-/- into which human SAP had been injected and SAP-/- into which mouse SAP had been injected. There was no significant difference in clearance rates between any group of mice in either experiment.

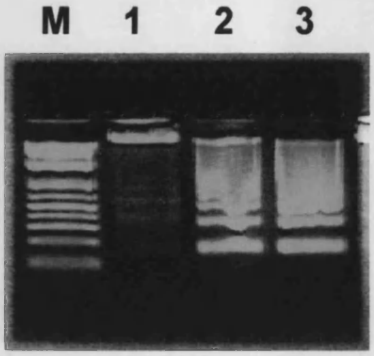

Figure 4.8 DNA extraction and agarose gel electrophoresis of two separate batches of chromatin. M is a 1 kilobase marker. Lane 1 is the batch of chromatin used for all previous experiments and lanes 2 and 3 are the degraded chromatin from which the nucleosomes for the experiment presented in figure 4.7 were prepared by calcium precipitation.

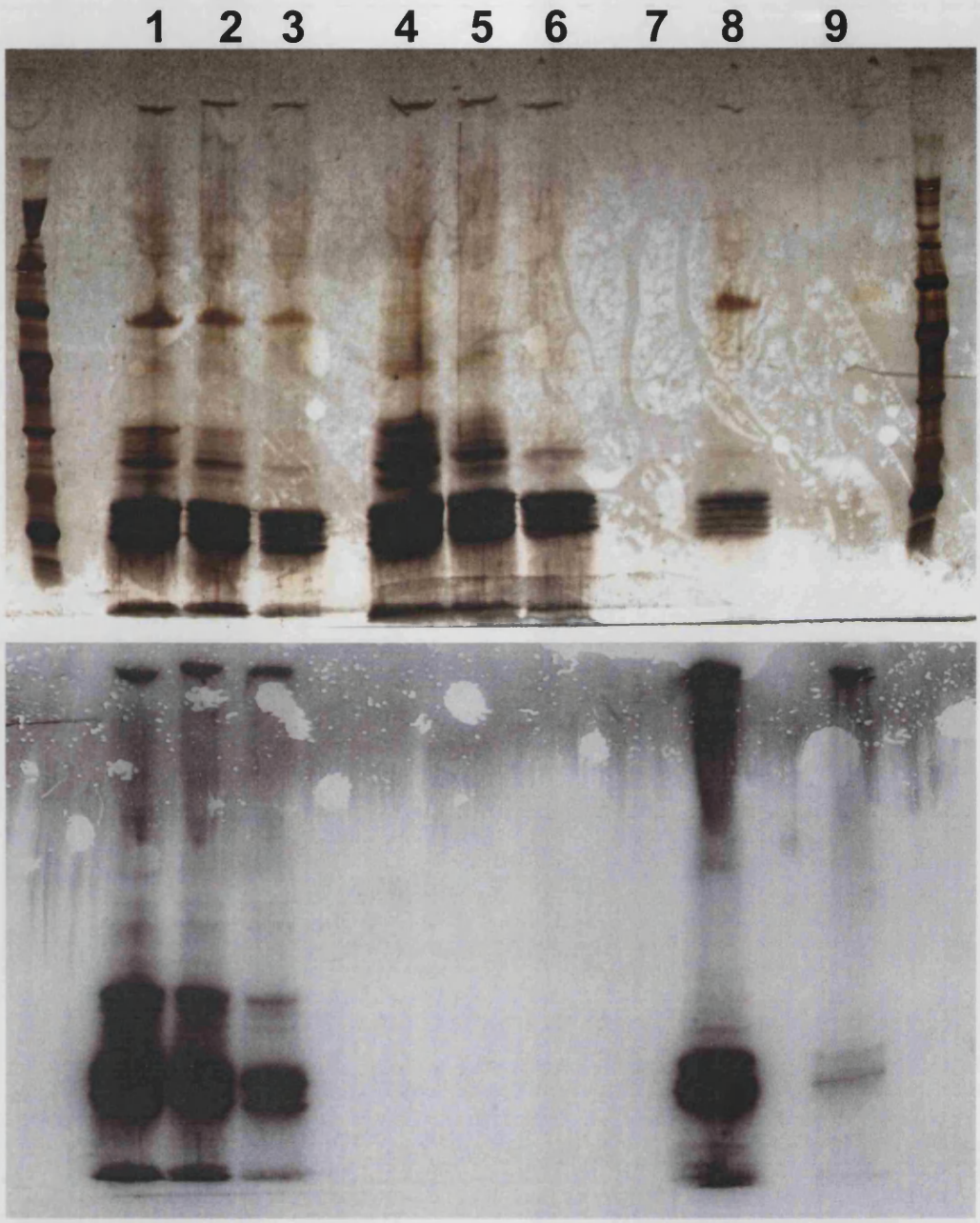

Figure 4.9 Autoradiography of SDS-PAGE confirming successful radioiodination of native (lanes 1-3) and tyramine cellobiose-labelled (lanes 7-9) nucleosomes. In the upper panel, SDS-PAGE stained with silver nitrate confirming the presence of the different histone components, including histones H1 and H5, in the nucleosome preparations. In the lower panel, autoradiography of the SDS-PAGE gel confirming successful radioiodination of both native and TC-labelled nucleosome components, with no radioactivity associated with unlabelled nucleosomes in lanes 4, 5, and 6. Three different volumes of each nucleosome preparation were loaded onto the gel.

removal from the plasma. The most sensitive single index of this is comparison of *in vivo* plasma decay kinetics between the TC-labelled and native proteins. Figure 4.10 displays the identity between plasma decay kinetics of TC-labelled and directly radioiodinated nucleosomes, thereby validating as physiological, the use of the TC-labelled nucleosome preparation for examination of sites of catabolism of nucleosomes.

Twenty-four hours after injection, nucleosome clearance from the plasma was complete. The liver was the main site of catabolism of nucleosomes, followed by the kidneys accounting for 65.1% (+/- 8.0) and 14.1% (+/- 1.7), respectively. Retrieval from the carcass was 16.5% (+/- 9.8%) and from the spleen and intestine were 0.9% (+/- 0.3) and 0.7% (+/- 0.1) respectively. The remaining organs each accounted for catabolism of $< 0.5\%$ of injected nucleosomes. There was no significant difference in overall contribution to catabolism by any organ between SAP-/- and wild-type mice. These results are shown in figure 4.11.

Since the presence of SAP enhanced rapid clearance of nucleosomes from the plasma with a significant difference in the percentage of an injected dose cleared by 15 min, organ localisation of nucleosomes was compared at this time point between SAP-/- and wild-type C57BL/6 mice. In two separate experiments, the proportion of nucleosomes catabolised by the spleen at this time point was significantly higher in SAP-/- than wild-type mice ($P < 0.003$, Mann-Whitney U test) (table 4.1). Although

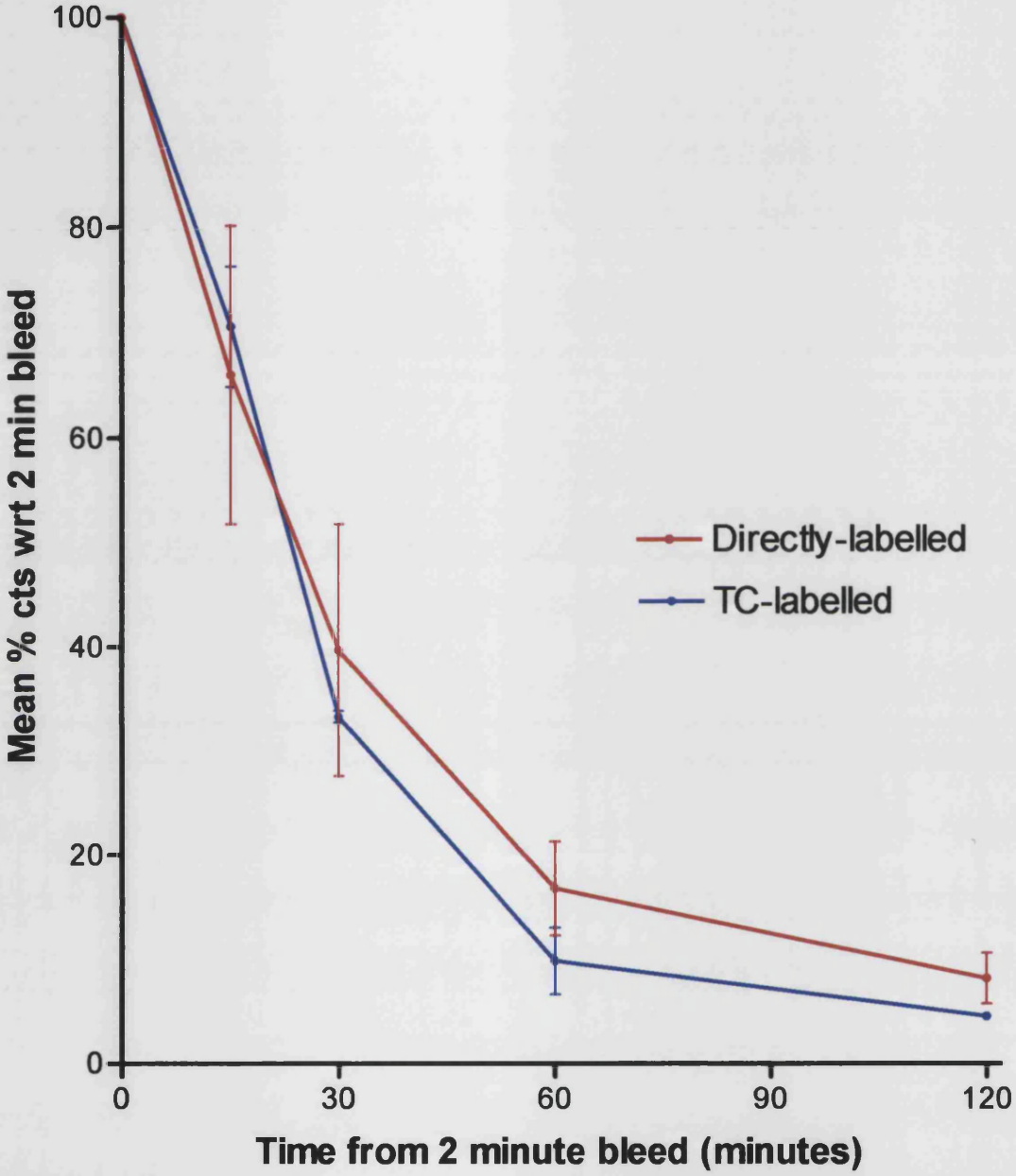

Figure 4.10 Comparison of in vivo plasma decay kinetics between directly radioiodinated and ^{125}I-tyramine-cellobiose (TC) labelled nucleosomes. There was no difference in clearance kinetics, thereby validating as physiological, the use of ^{125}I-tyramine-cellobiose labelled nucleosomes for determination of organ localisation of nucleosome catabolism.

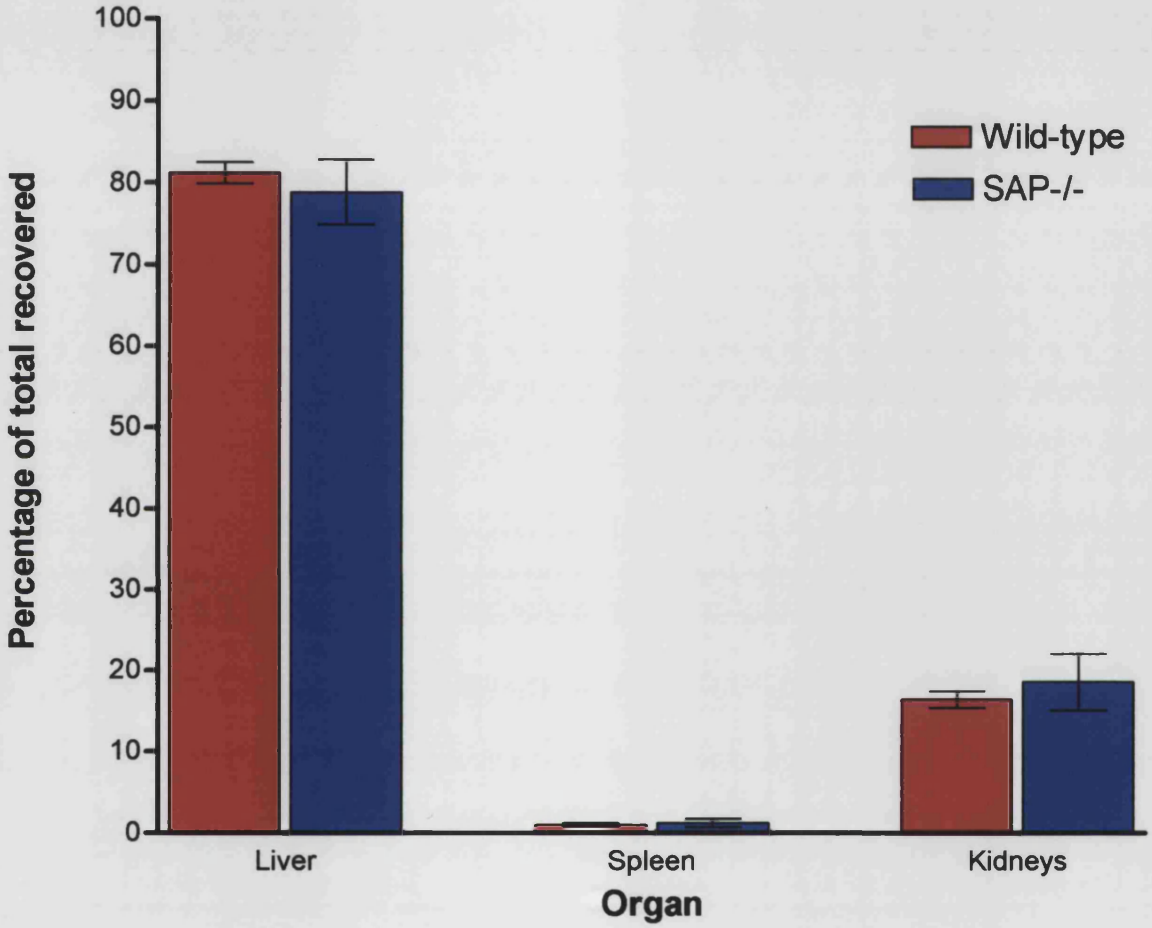

Figure 4.11 Comparison of organ localisation of catabolism of nucleosomes between SAP deficient and wild-type C57BL/6 mice. The major sites of catabolism of nucleosomes were the liver and kidneys. There was no difference between genotypes in the organs in which nucleosomes were catabolised.

the contribution to catabolism by the spleen was comparatively small, it was nearly twice as great in SAP-/- than wild-type mice.

Dipping film autoradiography was carried out on selected sections of liver, kidney and spleen from 3 wild-type and 3 SAP-/- mice, as described in Materials and Methods. Catabolism took place in the hepatocytes and Kupffer's cells of the liver (figure 4.12a), in the red pulp of the spleen (figure 4.12b) and in the parenchymal cells of the kidney (figure 4.12c). There was no discernible difference between SAP-/- and WT mice in the cellular localisation of nucleosome catabolism.

SUMMARY

Understanding the clearance and processing of native long chromatin and nucleosomes, believed to be the principal immunogens in SLE [269-274], is central to understanding the pathogenesis of the disease.

The clearance and processing of nuclear constituents such as nucleosomes and chromatin has not been studied in detail. The "trapped catabolism method," in which tracer is trapped in the cells in which the bound protein is catabolised, has not previously been used for determining the site of nucleosome catabolism. Using this method we have identified hepatocytes and Kupffer's cells in the liver and renal parenchymal cells as the major sites of nucleosome catabolism, accounting for approximately 97.5% of the total.

A)

	Liver	Spleen	Kidneys	Carcass
	Mean percentage (+/- SD)			
Wild-type	38.8 (7.8)	0.6 (0.2)	6.4 (1.2)	46.4 (7.8)
SAP-/-	32.7 (11.7)	1.0 (0.3)	7.2 (1.4)	45.2 (7.4)
P value	> 0.05	< 0.003	> 0.05	0.05

B)

	Liver	Spleen	Kidneys	Carcass
	Mean percentage (+/- SD)			
Wild-type	42.9 (3.1)	0.6 (0.1)	7.2 (0.7)	40.2 (3.6)
SAP-/-	40.3 (5.3)	1.0 (0.3)	7.4 (1.0)	42.7 (4.1)
P value	> 0.05	< 0.001	> 0.05	> 0.05

Table 4.1 Organ contribution to catabolism of nucleosomes within 15 minutes of intravenous injection. In both experiments A) and B), a significantly higher proportion of the nucleosomes were catabolised in the spleens of SAP-/- mice compared to wild-type mice at this timepoint ($P < 0.003$ and $P < 0.001$, respectively, Mann-Whitney U test). There was no other difference between genotypes.

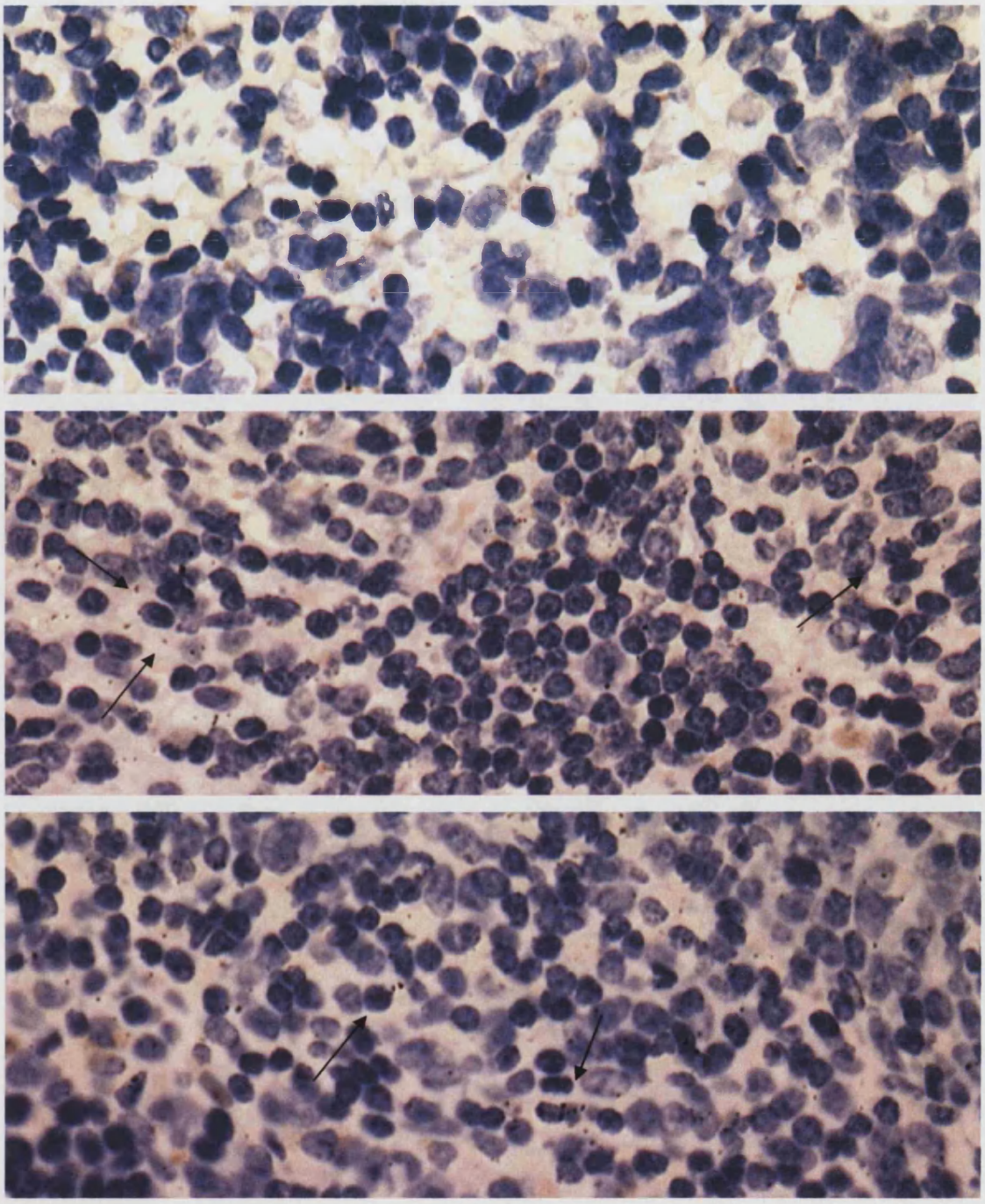

Figure 4.12A) Splenic histology and dipping film autoradiography from three mice. The upper panel is from a wild-type mouse into which unlabelled nucleosomes were injected. The middle and lower panels are from SAP-/- and wild-type mice respectively, into which ^{125}I-tyramine-cellobiose labelled nucleosomes were injected. Radioactivity is seen in the red pulp in the wild-type and SAP-/- mice with no difference in cellular location (arrowed)

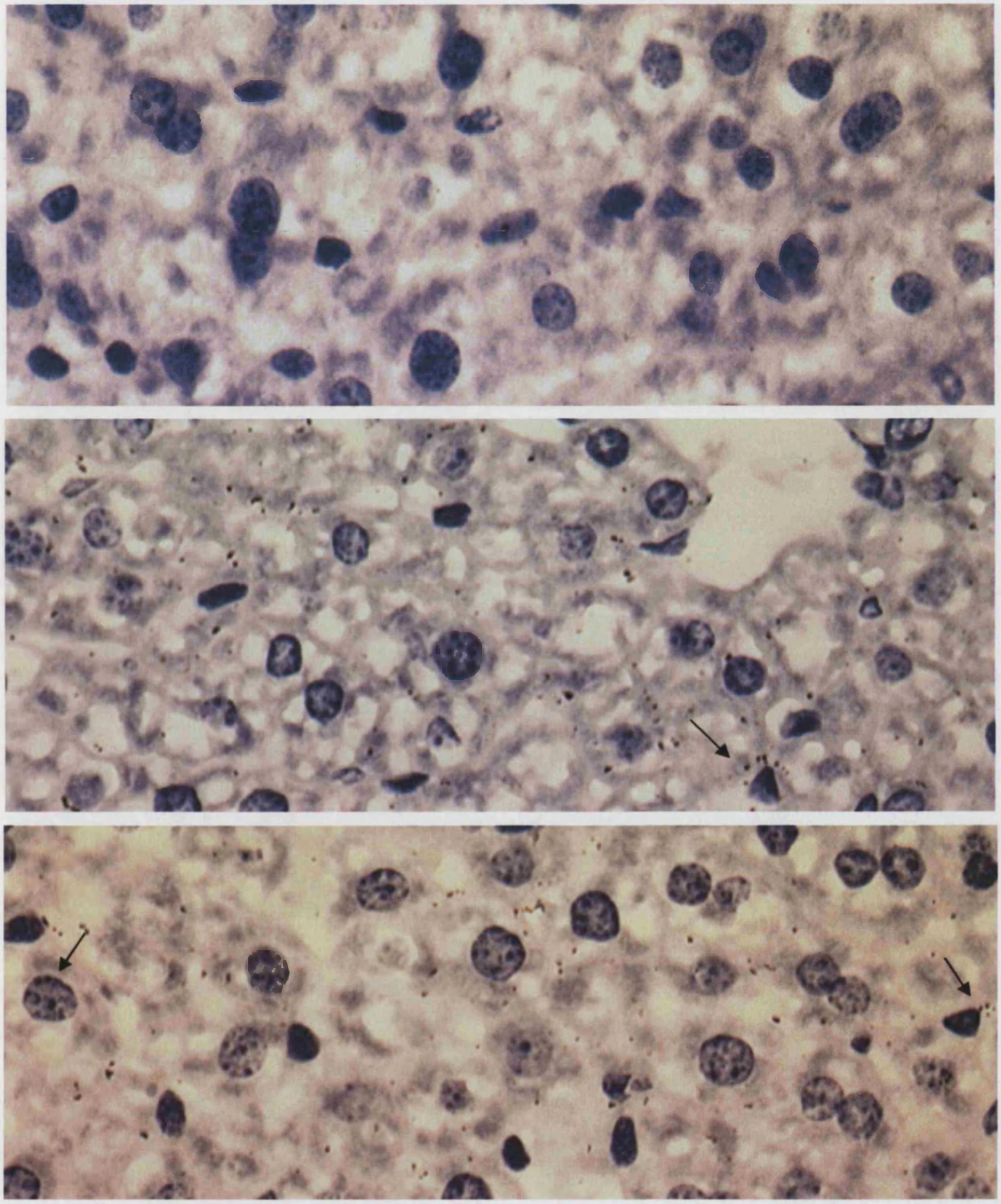

Figure 4.12B) Liver histology and dipping film autoradiography from a wild-type mouse injected with unlabelled nucleosomes (top panel) as a negative control. ^{125}I-tyramine-cellobiose labelled nucleosomes were injected into an SAP-/- mouse (middle panel) and a wild-type mouse (lower panel). Radioactivity was identified in Kuppfer's cells and in hepatocytes (arrowed) with no difference in distribution between SAP-/- and wild-type mice

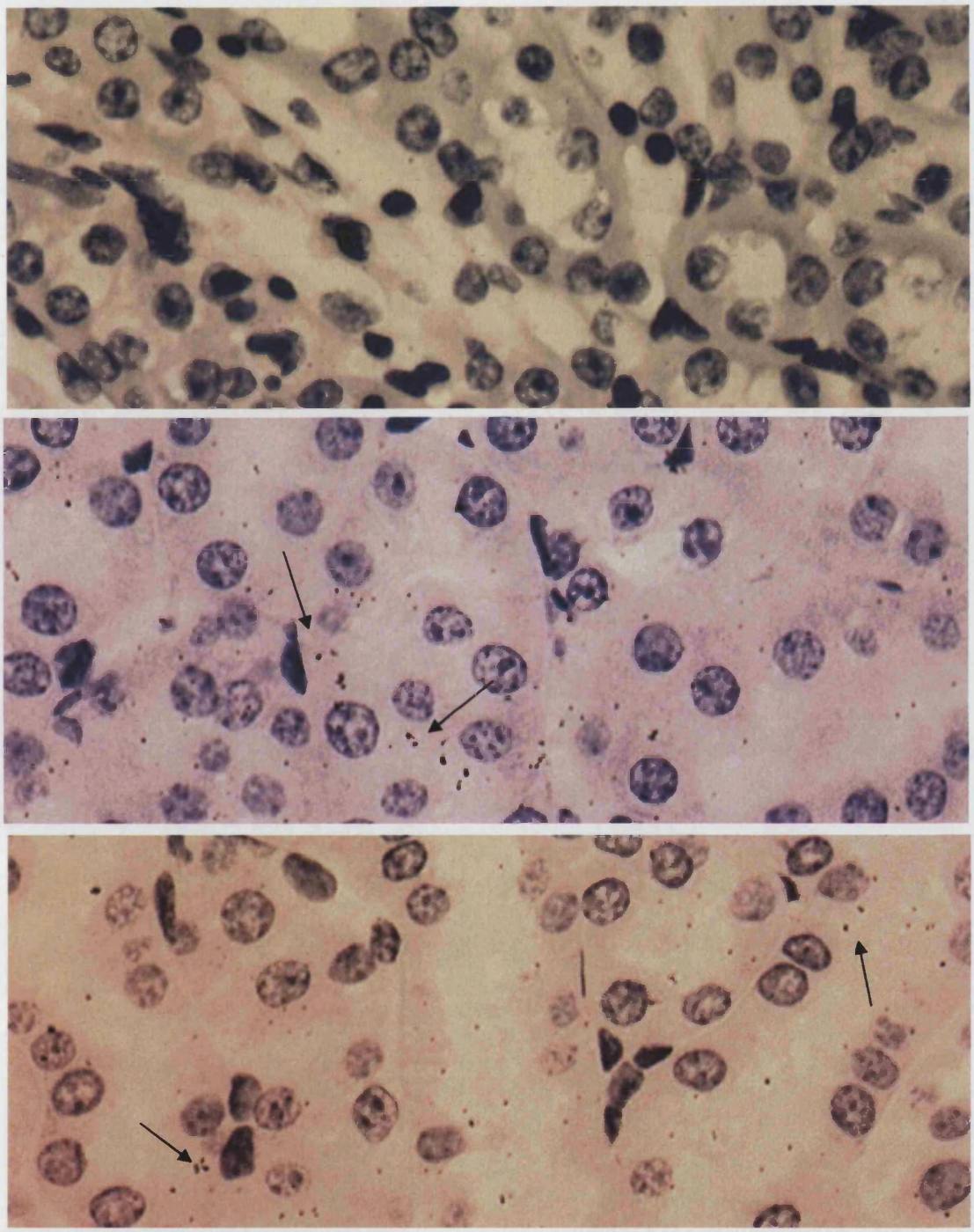

Figure 4.12C) Renal histology and dipping film autoradiography from a wild-type mouse injected with unlabelled nucleosomes (top panel) as a negative control. 125I-tyramine-cellobiose labelled nucleosomes were injected into an SAP-/- mouse (middle panel) and a wild-type mouse (lower panel). Radioactivity was identified in parenchymal cells of the kidney (arrowed) with no difference in distribution between SAP-/- and wild-type mice

The discovery that SAP is the main DNA, histone and chromatin binding protein of the plasma [61], in conjunction with the spontaneous SLE-like phenotype in SAP-/- C57BL/6 mice reported here, suggested that SAP might have an important role in the *in vivo* handling of nucleosomes and/or long chromatin. In order to investigate this possibility, we compared sites of catabolism of nucleosomes and plasma decay kinetics of injected avian chromatin and nucleosomes between SAP-/- and wild-type C57BL/6 mice.

Although the overall contribution to catabolism of nucleosomes by the spleen was comparatively small, within 15 min of injection it was nearly twice as great in SAP-/- mice compared to that in wild-type mice ($P < 0.003$, Mann-Whitney U test). Interestingly however, by 24 h there was no difference between genotypes in proportion of nucleosomes catabolised by the spleen. Previous studies in mice have suggested that approximately 0.5% of an injected antigen is processed by the spleen [344,345]. However, the time course of such processing is unknown. It is possible that in the absence of SAP, rapid uptake and processing of nucleosomes by the spleen resulting in a transiently high concentration of antigen is sufficient to initiate immunogenic rather than tolerogenic processing.

The present work demonstrates that absence of SAP significantly delayed clearance of both long chromatin and nucleosomes from the plasma. This was unexpected since previous experiments from our laboratory, using whole body counting of radioactivity, had shown accelerated clearance of long chromatin in SAP-/- mice

compared to wild-type animals, with significantly increased whole-body counts in wild-type mice 4 h after injection. However, whole body counting of radioactivity and plasma clearance examine different physiological processes; furthermore whole body counting is susceptible to inaccuracies due to variable bladder voiding and variable contamination of the fur with excreted tracer.

That SAP accelerates clearance from the plasma but decelerates whole body clearance of long chromatin, is far from inconceivable. The fast removal of chromatin from the plasma in the presence of SAP may reflect binding of the SAP portion of the SAP-chromatin complex to sites of clearance, thereby enhancing uptake of chromatin from the plasma into relevant organs. The presence of a specific receptor for chromatin on hepatocytes or Kupffer's cells has not been demonstrated. One might speculate that upon uptake of SAP-coated chromatin into the catabolising organ, SAP, which is itself known to be highly resistant to proteolysis [68,204], slows catabolism of the bound chromatin, thereby delaying whole body clearance.

The mechanisms by which persistence of chromatin and nucleosomes in the circulation initiate or influence development of autoimmunity are unknown at present. The possibility that the delayed plasma clearance of chromatin and nucleosomes in SAP-/- mice in itself represents uptake by circulating antigen presenting cells was excluded by the fact that counting was performed on TCA precipitates of plasma. One might expect that any persistence in the circulation of

SAP deficient mice might increase the chance of initiating an autoimmune response. Alternatively however, the binding of SAP to long chromatin and nucleosomes may prevent initiation of autoimmunity not only by increasing the speed of their removal from the circulation, but by shielding the bound nuclear constituents from the immune system, analogous to the role of SAP in host defence, in which certain bacteria appear to have adapted to bind SAP, thereby preventing their recognition [69].

This work demonstrates also the difficulty of working with chromatin and nucleosomes. The chicken erythrocyte chromatin was prepared and stored in several batches as described in Materials and Methods. Nucleosomes were prepared using chromatin as the starting material by exactly the same method prior to each group of experiments. However, comparison of different batches of chromatin by agarose gel electrophoresis showed that there was significant degradation of the starting material over time. Since long chromatin consists of a series of tightly packed nucleosomes which in turn, consist of histones associated with DNA, it is almost impossible to determine whether the material with which one is working is behaving physiologically.

SAP therefore, appears to influence both the rate of plasma clearance of nuclear constituents, and the time course of their splenic uptake and catabolism. However, the detailed mechanisms by which events in the circulation, into which nucleosomes

are released by cell apoptosis, and events in the spleen, in which nucleosomes are processed and degraded, initiate systemic autoimmunity remain to be elucidated.

Chapter 5 - Pentraxins And Apoptosis

INTRODUCTION

There is much evidence that the autoimmune response in SLE is driven by antigen and that the initial immunogen consists of DNA-protein complexes. There is also evidence that anti-DNA antibodies cross react with small nuclear ribonuclear proteins (snRNPs) and that these may be potential immunogens [346]. Apoptotic cells have emerged in recent years as likely sources of these autoantigens which are ubiquitous and abundant intracellular components of all healthy tissues in the body.

Cell death is a key feature of growth, development and repair and normally occurs by the mechanism of apoptosis, or programmed cell death. Under normal circumstances, apoptotic cells are efficiently phagocytosed by other cells by means of a variety of specific receptors [276,347-349]. Such rapid clearance of apoptotic cells is believed to be important in inhibiting inflammation and autoimmune responses against intracellular antigens [322,350].

Recognition of the possible source of the autoantigens of SLE first came with the observation by Casciola-Rosen *et al* that many of the most characteristic "intracellular" autoantigens of SLE were present in surface blebs on cells undergoing apoptosis [277]. This led to the hypothesis that it might be apoptotic

cells and bodies that are the source of the autoantigens that drive the autoimmune response in SLE.

This hypothesis is supported by considerable experimental evidence. Several of the mouse models of SLE result from mutated genes encoding proteins which regulate development of apoptosis [238,239]. For example, the presence of the *lpr* gene, which encodes a mutated *fas* gene, dramatically enhances susceptibility to lupus in the MRL strain of mouse [238]. Intravenous administration into normal mice of syngeneic apoptotic thymocytes was shown by Elkon's group to induce autoantibody production [278]. Furthermore, defective *in vivo* clearance of injected apoptotic thymocytes has been demonstrated in lupus-prone C1q-deficient mice [279]. Among humans with SLE, both increased lymphocyte apoptosis [280] and, more recently defective clearance of apoptotic cells [281,351] have been proposed to be important events in the aetiopathogenesis of the disease.

One possible reason for enhancement of the immunogenicity of various potential autoantigens in SLE, is that during apoptosis they become modified [282]. In particular, apoptosis induced by the action of granzyme B was shown to modify many of the targets of systemic immune responses [283], and it is proposed that apoptosis might thus render cryptic epitopes immunodominant and lead to immunogenic presentation of epitopes to which the immune system has not achieved tolerance [284].

Although CRP was shown to bind to nuclei and chromatin *in vitro* [296,297], Pepys' group demonstrated that SAP and CRP bind to different nuclear structures *in vivo.* SAP is the major calcium-dependent specific DNA binding protein in the serum under physiological conditions [60-62] and CRP binds to nuclei via snRNPs [62]. Experiments using sera from various animals (mouse, rat, guinea pig, cow and plaice) all demonstrated specific calcium-dependent binding of SAP to DNA. Isolated purified human SAP also showed major calcium-dependent binding to native long chromatin, H1-stripped chromatin and native DNA at physiological ionic strength, and to nucleosomes in solution. Binding of SAP to chromatin was specifically shown to displace H1-type histones and solubilise long chromatin [61]. SAP was also shown to bind to dermal keratin bodies, that is, apoptotic cells, in normal skin [300] and to chromatin released from necrotic cells *in vivo* [299].

Since intravenous administration of syngeneic apoptotic cells had been shown to induce antinuclear autoantibody production in normal mice [278], we wondered whether SAP deficiency, which spontaneously leads to ANA production, glomerulonephritis and an enhanced immune response to chromatin, might enhance the immunogenicity of apoptotic cells. The studies presented here examine the possible role of pentraxins in handling apoptotic cells. The binding of SAP to apoptotic cells was confirmed and CRP binding to apoptotic cells was demonstrated. The immune response to injection of syngeneic apoptotic thymocytes was compared in SAP-/- and wild-type mice *in vivo,* and a possible effect of SAP on macrophage ingestion of apoptotic lymphocytes was sought both *in vitro* and *in vivo.*

BINDING OF PENTRAXINS TO APOPTOTIC CELLS

Binding of SAP to Apoptotic Jurkhat Cells

All cells were stained with the DAPI counterstain. Approximately 80% of cells that had been exposed to UV irradiation were apoptotic according to the TUNEL assay after 6 h. SAP bound to the surface of apoptotic Jurkhat cells, as determined by colocalisation of staining with anti-human SAP and TUNEL staining (figure 5.1A). Cells that had not been exposed to UV irradiation showed < 5% staining by TUNEL assay and SAP did not bind to TUNEL-negative cells. Binding of SAP was completely calcium dependent, indicating that specific recognition by SAP of particular cell surface ligands was involved. The specificity of the RITC-anti-human SAP antibody for SAP was confirmed by complete abolition of staining after pre-absorption of the antibody with immobilised pure SAP (figure 5.1B).

Binding of CRP to Apoptotic Jurkhat Cells

Approximately 88% of Jurkhat cells that were incubated with a monoclonal anti-Fas antibody were FITC-annexin V positive after 6 h. Exclusion of trypan blue confirmed viability in over 95% of cells at this time point. CRP bound to approximately 45% of cells that had been incubated with the anti-Fas monoclonal antibody (Figure 5.2). There was no binding of CRP to the "non-apoptotic" cell population that had not been incubated with the anti-Fas monoclonal antibody. Binding of CRP was completely calcium dependent, indicating that specific recognition by CRP of particular cell surface ligands was involved. The specificity of the FITC-anti-human CRP antibody

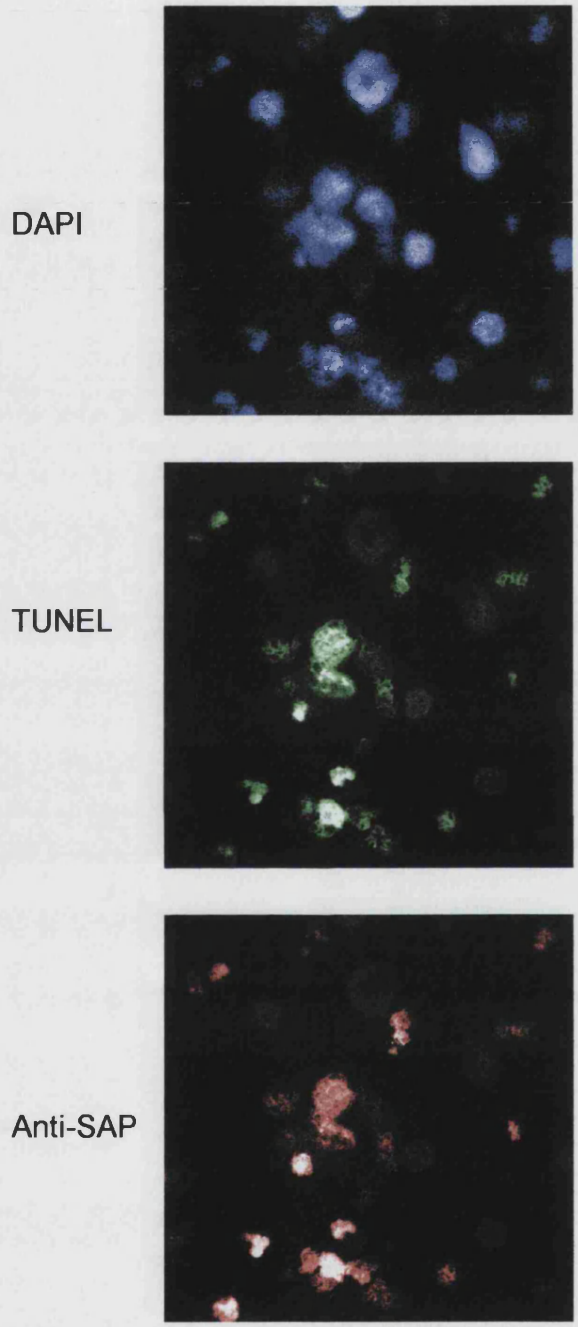

Figure 5.1A Binding of SAP to apoptotic Jurkhat cells viewed by confocal microscopy. The DAPI counterstain (upper panel) shows all cells, the green fluorescence (middle panel) show cells which are apoptotic by TUNEL staining and the red fluorescence (lower panel) shows anti-SAP staining. SAP bound to the surface of apoptotic cells but not to non-apoptotic cells.

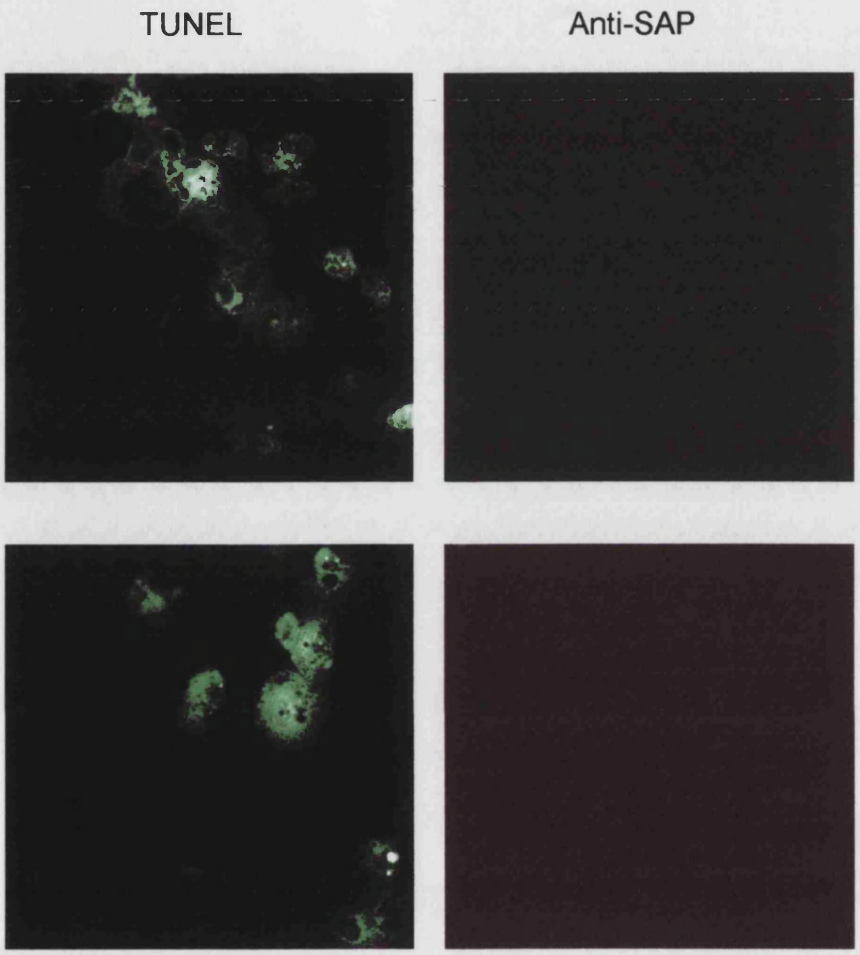

Figure 5.1B Binding of SAP to apoptotic cells - control samples. The upper panels confirm specificity of SAP binding and show TUNEL staining (left) and absence of staining with anti-SAP (right) after incubation of cells with SAP in the presence of 10 mM EDTA. The lower panels confirm specificity of the anti-SAP antibody for SAP and show TUNEL staining (left) and absence of staining with anti-SAP after solid phase pre-absorption of the antibody with pure antigen.

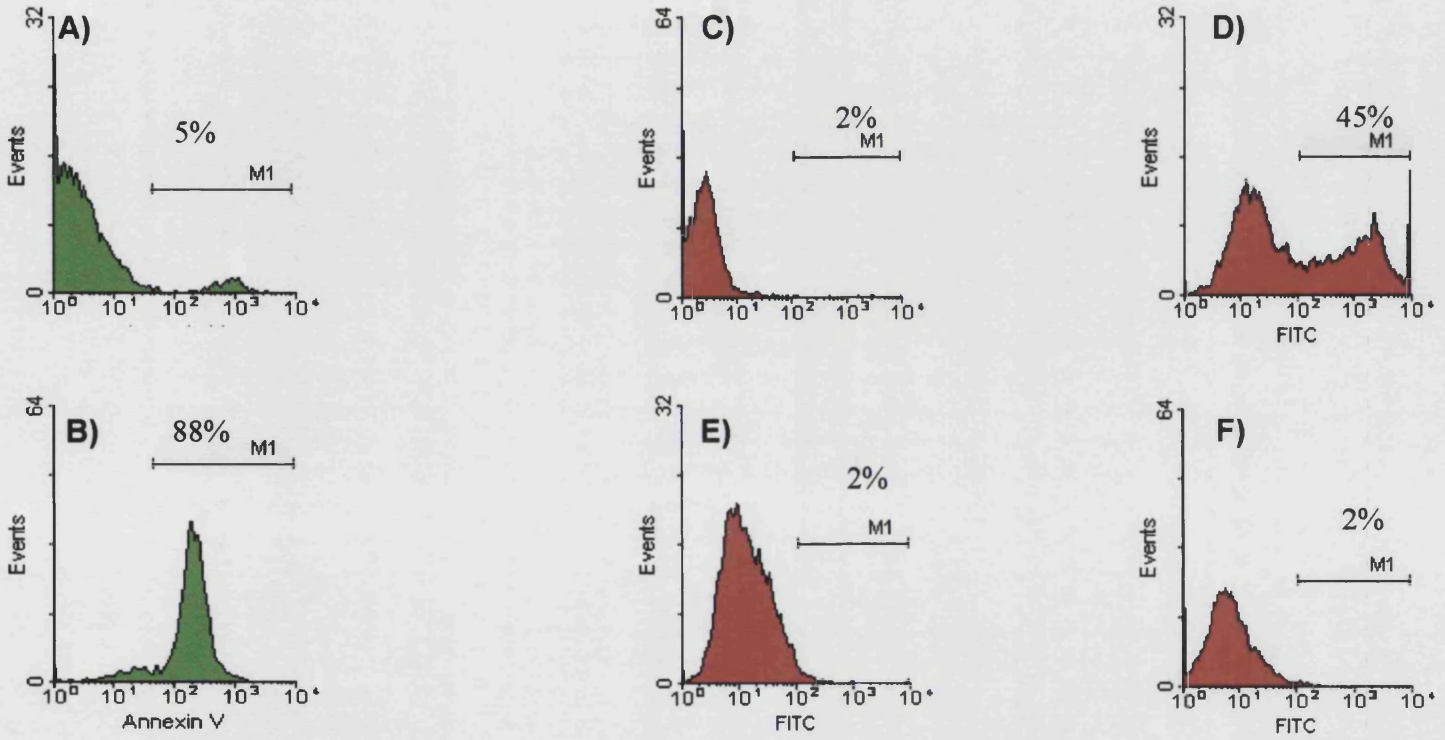

Figure 5.2 Binding of CRP to apoptotic Jurkhat cells shown by flow cytometry. A-B) Apoptosis induction A) Absence of apoptosis (as defined by annexin V binding) among cells incubated in the absence of anti-Fas monoclonal antibody B) Presence of apoptosis (defined by annexin V binding) in 88% of Jurkhat cells after incubation for 6 hours in the presence of anti-Fas monoclonal antibody. C-G) Binding of CRP to Jurkhat cells C) Absence of CRP binding to non-apoptotic Jurkhat cells D) Binding of CRP to 45% of all cells 6 hours after induction of apoptosis. E) Absence of CRP binding to apoptotic Jurkhat cells after incubation with CRP in the presence of EDTA, confirming calcium dependence of CRP binding F) Absence of fluorescence after incubation of apoptotic Jurkhat cells with CRP and staining with fluorochrome labelled antibody, from which the anti-CRP had been specifically removed by prior incubation with pure CRP, confirming specificity of the fluorescent antibody for CRP.

for CRP was confirmed by complete abolition of staining after pre-absorption of the antibody with immobilised pure CRP, as shown by flow cytometric analysis in figure 5.2.

Competition between SAP and CRP for Apoptotic Cell Binding

Apoptosis occurred in 89% of all cells after 6 h of incubation with anti-Fas antibody and SAP binding was detected on 76-82% of all cells. There was no reduction of SAP binding in the presence of high concentrations of CRP. Cells incubated with CRP alone did not stain with anti-SAP (figure 5.3).

After 6 h of incubation with anti-Fas antibody, 92% of cells were apoptotic as defined by annexin V binding and CRP binding was detected on 38-52% of all cells. There was no reduction of CRP binding in the presence of high concentrations of SAP. Cells incubated with SAP alone did not stain with anti-CRP. These experiments demonstrate that there is no competition between SAP and CRP for binding to ligands on the surface of apoptotic cells, and presumably the two pentraxins recognize different molecules in this situation, just as they do in intact nuclei [62].

IMMUNE RESPONSE TO ADMINISTRATION OF SYNGENEIC APOPTOTIC THYMOCYTES

Comparison between SAP-/- and Wild-type C57BL/6 Mice

ANAs were not detected prior to the first injection of apoptotic cells in any C57BL/6 mice regardless of sex or SAP genotype. On day 14 (2 weeks after the first injection of

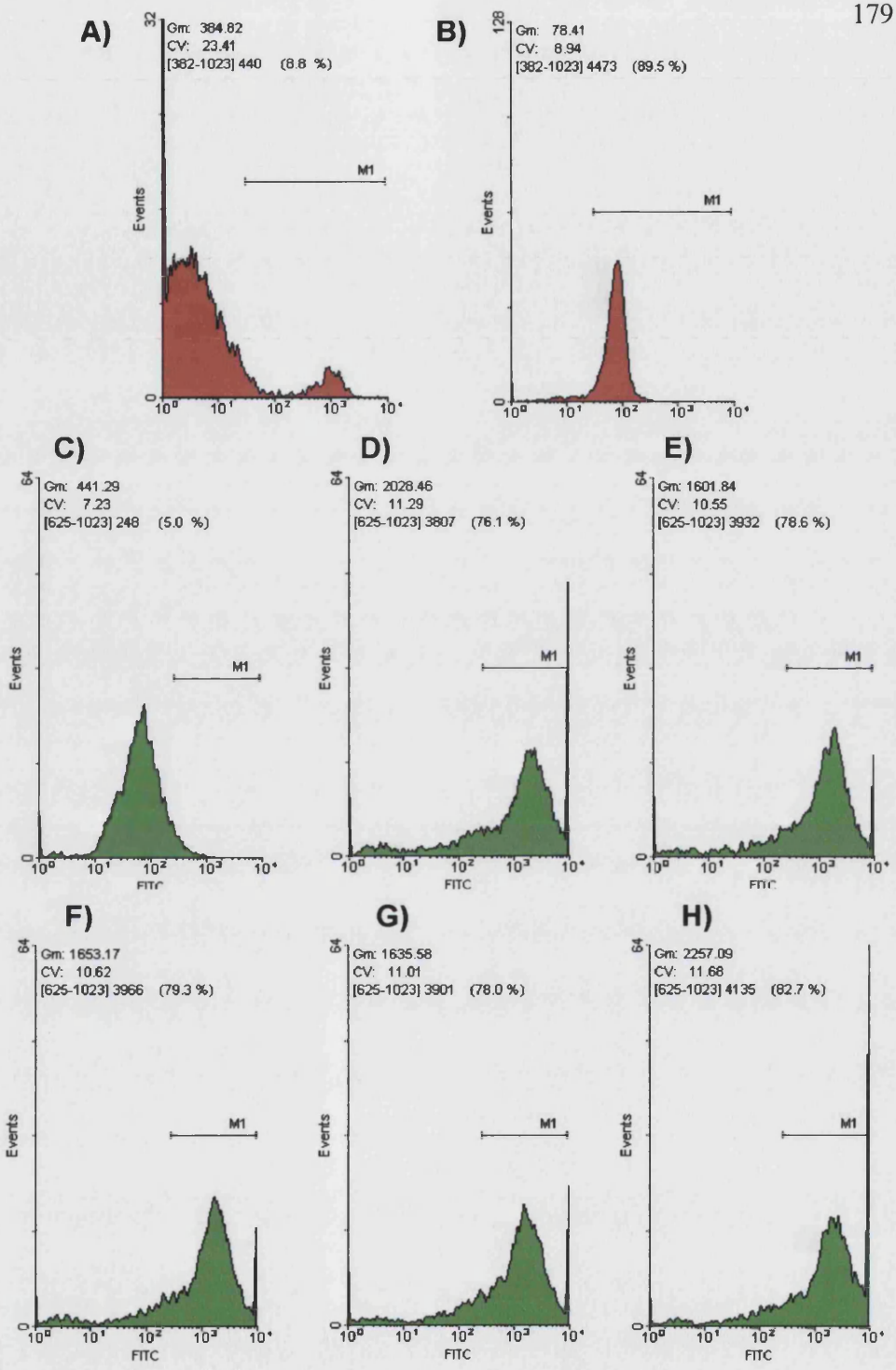

Figure 5.3 Binding of SAP to the surface of apoptotic Jurkhat cells in the presence of increasing concentrations of CRP. A-B) Induction of 90% Jurkhat cell apoptosis by incubation with anti-Fas monoclonal antibody for 6 hrs C) Absence of SAP binding to apoptotic Jurkhat cells after incubation with CRP alone D-H) SAP binding to apoptotic Jurkhat cells after incubation with D) SAP alone E) SAP and 1 mg/l CRP F) SAP and 10 mg/l CRP G) SAP and 100 mg/l CRP H) SAP and 1000 mg/l CRP

apoptotic cells), ANAs were present in 0/20 female wild-type mice compared to 7/26 female SAP-/- mice ($P < 0.02$, Fisher's exact test), with titres of 1/80 in 5 animals, and 1/160 and 1/320 in the other two. ANAs were present in 0/10 male wild-type mice compared to 2/9 male SAP-/- mice ($P > 0.05$, Fisher's exact test), with titres of 1/80.

On day 28, ANAs were present in 1/20 female (at a titre of 1/80) and 0/10 male wild-type mice compared to 18/26 female and 3/9 male SAP-/- mice (females: $P < 0.0001$; males: $P > 0.05$, Fisher's exact test). The median ANA titre in female SAP-/- mice was 1/80 (range; 0-1/1280). On day 28, antibodies against dsDNA, ssDNA and chromatin were determined in all mice and results are shown in table 5.1. Anti-ssDNA antibodies were present in 16/26 SAP-/- females compared to 5/20 wild-type females ($P < 0.02$, Fisher's exact test) and anti-chromatin antibodies were present in significantly more female SAP-/- (20/26) than female wild-type (9/20) mice at this time point ($P < 0.04$, Fisher's exact test). There were no significant differences in autoantibody titre between male mice of the two genotypes.

On day 42, ANAs were present in 1/20 female (titre of 1/80) and 0/10 male wild-type mice compared to 25/26 female and 3/9 male SAP-/- mice (females: $P < 0.0001$, Fisher's exact test). Interestingly, there was a rise in ANA titres between days 28 and 42 in 14 female and 1 male SAP-/- mice, ANA titres remained unchanged in 8 female and 6 male SAP-/- mice and fell from day 28 to day 42 in 4 female and 2 male SAP-/- mice. The median ANA titre in female SAP-/- mice on day 42 was 1/160, compared to

		Number	Anti-nuclear antibody	Anti-double stranded DNA	Anti-chromatin	Anti-single stranded DNA	Glomerulonephritis
			Number (%) +ve	Number (%) +ve	Number (%) +ve	Number (%) +ve	Number (%) +ve
Female	Wild-type	20	1 (5)	4 (20)	9 (45)	5 (25)	0 (0)
	SAP-/-	26	18 (69)	9 (35)	20 (77)	16 (62)	9 (36)
			P < 0.0001	P = 0.33	P < 0.04	P < 0.02	P < 0.003
Male	Wild-type	10	0 (0)	2 (20)	6 (60)	2 (20)	0 (0)
	SAP-/-	9	3 (33)	4 (44)	4 (44)	2 (22)	0 (0)
			P = 0.09	P = 0.35	P = 0.36	P = 1.0	P = 1.0

P values obtained by Chi-squared or Fisher's exact test as appropriate

Table 5.1 Comparison of autoimmunity and glomerulonephritis between wild-type and SAP-/- C57Bl/6 mice following intravenous administration of 1 x 10^7 apoptotic syngeneic thymocytes weekly, for 4 weeks. Autoimmunity is compared on day 28 (4 weeks after 1st intravenous injection of apoptotic thymocytes) and glomerulonephritis was determined on day 56.

1/80 on day 28. The median ANA titres were negative in male SAP-/- mice on both day 28 and day 42.

Glomerulonephritis was present in 9/26 (35%) female and 0/9 male SAP-/- mice and but was absent in all wild-type mice (females: $P < 0.003$, Fisher's exact test) 56 days after the first injection. Glomerulonephritis was grade I in 5 mice and grade II and III in 2 cases each.

Comparison between Wild-type, SAP-/- and SAP-/-, hSAP Transgenic C57BL/6 Mice

ANAs were not detected prior to the first injection of apoptotic cells in any mouse regardless of genotype. On day 14 (2 weeks after the first injection of apoptotic cells), ANAs were present in 0/10 wild-type mice compared to 7/16 SAP-/- mice and 4/16 SAP-/-, hSAP Tg mice ($P < 0.03$, SAP-/- vs wild-type; $P > 0.05$ wild-type vs SAP-/-, hSAP Tg, Fisher's exact test).

Presence of autoantibodies on day 28 are shown in table 5.2. The median ANA titre in SAP-/- and SAP-/-, hSAP Tg mice at this time point was 1/80 (range; 0-1/1280).

On day 42, ANAs were present in 0/10 wild-type mice, 16/16 SAP-/- mice and 11/16 SAP-/-, hSAP Tg mice ($P < 0.0001$, wild-type vs SAP-/-; $P < 0.0008$, wild-type vs SAP-/-, hSAP Tg; $P > 0.05$ SAP-/- vs SAP-/-, hSAP Tg, Fisher's exact test). There was a rise in ANA titres between days 28 and 42 in 8 SAP-/- mice and 7 SAP-/-, hSAP Tg mice, ANA titres remained unchanged in 6 SAP-/- mice and 8

	N	Anti-nuclear antibody	Anti-double stranded DNA	Anti-chromatin	Anti-single stranded DNA	GN
		Number (%) +ve	Number (%) +ve	Number (%) +ve	Number (%) +ve	Number (%) +ve
Wild-type	10	0 (0)	0 (0)	2 (20)	3 (30)	0 (0)
SAP-/-	16	12 (75)	2 (12)	11 (69)	13 (81)	8 (50)
SAP-/- hSAP Tg	16	11 (69)	1 (6)	8 (50)	12 (75)	13 (81)
P value (SAP-/- vs wild-type)		0.0002	0.5	0.04	0.02	0.009
P value (SAP-/-, hSAP Tg vs wild-type)		0.0008	1.0	0.2	0.04	0.0001
P value (SAP-/- vs SAP-/-, hSAP Tg)		1.0	1.0	0.47	1.0	0.13

P values obtained by Chi-squared or Fisher's exact test as appropriate

Table 5.2 Comparison of autoimmunity and glomerulonephritis between wild-type, SAP-/- and SAP-/-, hSAP transgenic C57Bl/6 female mice following intravenous administration of 1 x 10^7 apoptotic syngeneic thymocytes weekly, for 4 weeks. Autoimmunity was compared on day 28 (4 weeks after 1st intravenous injection of apoptotic thymocytes) and glomerulonephritis was determined on day 56. There was no significant difference in autoimmunity or glomerulonephritis between SAP-/- and SAP-/-, hSAP Tg mice. However, there was significantly more autoimmunity and glomerulonephritis in SAP-/- and SAP-/-, hSAP Tg mice than in wild-type mice.

SAP-/-, hSAP Tg mice and ANAs fell from day 28 to day 42 in 2 SAP-/- mice and 1 SAP-/-, hSAP Tg mouse. The median ANA titre increased in SAP-/- mice from 1/80 to 1/320 between days 28 and 42, and in SAP-/-, hSAP Tg mice from 1/80 to 1/160.

There was no significant difference between presence or severity of glomerulonephritis in SAP-/- and SAP-/-, hSAP Tg mice (table 5.2).

Comparison between SAP-/- and Wild-type 129/Sv Mice

ANAs were not detected prior to the first injection of apoptotic cells in any mouse regardless of sex or SAP genotype. Similarly, ANAs were absent in all mice on days 14, 28 and 42. There was no glomerulonephritis in any mouse on day 56.

MACROPHAGE INGESTION OF APOPTOTIC THYMOCYTES IN VIVO

Comparison between SAP-/- and Wild-type C57BL/6 Mice

Thirty min after intraperitoneal injection of apoptotic thymocytes, approximately 30% of peritoneal macrophages were ingesting a thymocyte. At this time point, there was no significant difference in percentage of macrophages ingesting apoptotic thymocytes between wild-type and SAP-/- C57BL/6 mice (figure 5.4, table 5.3). Ingestion by macrophages was unaffected by whether the thymocytes were obtained from SAP-/- or wild-type mice.

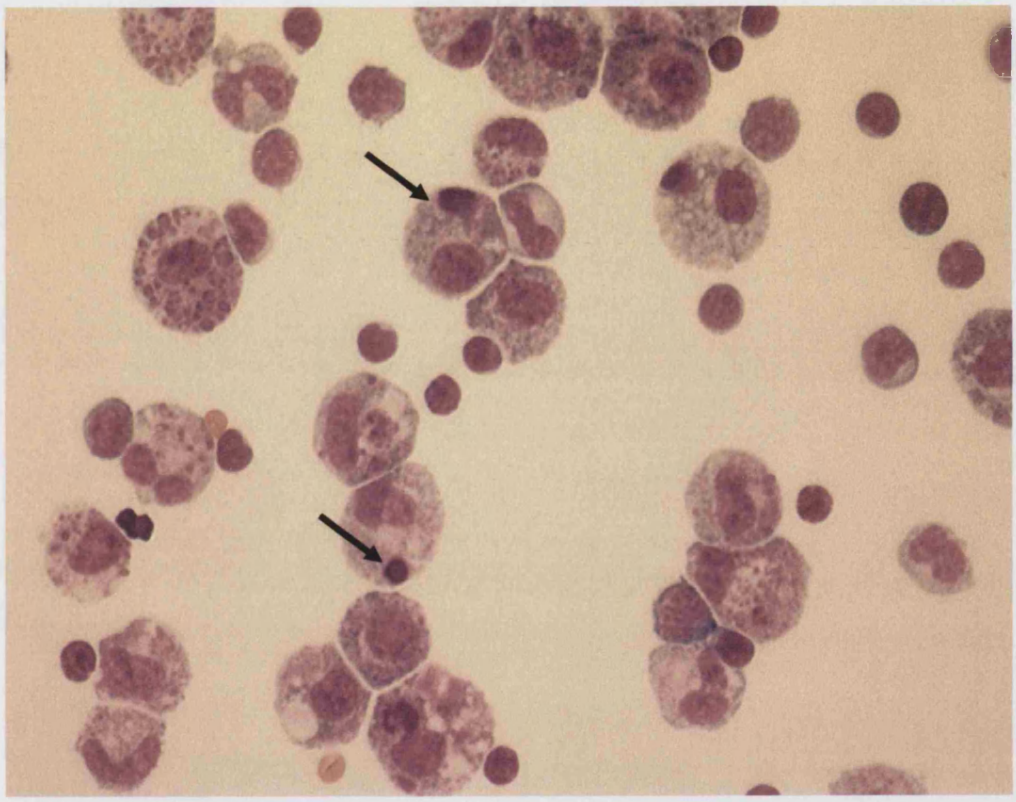

Figure 5.4 Ingestion of apoptotic mouse thymocytes by macrophages *in vivo*. Thirty minutes after intraperitoneal injection, small, darkly stained apoptotic thymocytes (arrowed) can be seen within the cytoplasm of certain peritoneal macrophages.

A)

	Experiment		
	1	2	3
Genotype injected	Mean (SD)	Mean (SD)	Mean (SD)
Wild-type	38 (9)	20 (9)	22 (11)
SAP-/-	35 (15)	22 (6)	30 (6)

P > 0.05, T-test

B)

	Experiment		
	1	2	3
Genotype injected	Mean (SD)	Mean (SD)	Mean (SD)
Wild-type	28 (14)	28 (5)	37 (10)
SAP-/-	30 (9)	36(6)	29 (14)

P > 0.05, T-test

Table 5.3 Percentage of SAP-/- and wild-type peritoneal macrophages ingesting apoptotic thymocytes. Each result is the mean and standard deviation from 6 mice. A) Comparison between SAP-/- and wild-type mice after injection of apoptotic thymocytes obtained from wild-type mice in three identically performed experiments. B) Comparison between SAP-/- and wild-type mice after injection of apoptotic thymocytes obtained from SAP-/- mice in three identically performed experiments. There was no difference between SAP-/- and wild-type animals in the percentage of macrophages ingesting apoptotic cells.

MACROPHAGE INGESTION OF APOPTOTIC HUMAN LYMPHOCYTES IN VITRO

Binding of SAP to the surface of apoptotic Jurkhat cells was confirmed prior to incubation with macrophages. SAP binding was absent after incubation of Jurkhat cells with SAP in the presence of EDTA.

Less than 4% percent of macrophages to which Jurkhat cells had not been added showed binding and/or ingestion of an apoptotic cell. Incubation of non-apoptotic Jurkhat cells with macrophages for 1 h resulted in binding and/or ingestion by 55% of macrophages. Presence of SAP on the surface of apoptotic Jurkhat cells was not found to significantly affect binding and/or ingestion by primary human macrophages (table 5.4). At least one SAP-coated apoptotic Jurkhat cell was bound and/or ingested by 88%, 75% and 61% of macrophages in three separate experiments, compared to 77%, 85% and 65% of macrophages respectively, which had bound and/or ingested a non SAP-coated apoptotic Jurkhat cell ($P > 0.05$, T-test).

SUMMARY

Previous studies have shown the binding of SAP to dermal keratin bodies *in vivo*, that is, apoptotic cells in human skin [300]. The studies presented here confirm the binding of SAP to the surface of apoptotic Jurkhat cells, and reveal that CRP, the classical acute phase reactant in humans, also binds to the surface of apoptotic cells *in vitro*. During

Incubated with Macrophages	Experiment 1	2	3
Apoptotic Jurkhat cells + SAP	88	75	61
Apoptotic Jurkhat cells - SAP	77	85	65
DMEM medium alone	3.5	4	3.5

SAP vs no SAP; $P > 0.05$, T-test

Table 5.4 Percentage of macrophages which had bound and/or ingested at least one apoptotic Jurkhat cell after incubation for 1 hour at 37°C. Comparison was between apoptotic Jurkhat cells coated with SAP on their surface (+ SAP) and Jurkhat cells on the surface of which there was no SAP (- SAP, by incubation in the presence of EDTA). Results from three identical experiments are shown. The presence of SAP on the surface of apoptotic Jurkhat cells did not significantly affect macrophage binding and/or ingestion at this timepoint. Incubation of macrophages with DMEM medium alone resulted in < 4% showing binding and/or ingestion of an apoptotic cell.

the course of these studies, Hack and colleagues reported calcium-dependent binding of SAP to apoptotic cells that was independent of binding to chromatin [352]. They postulated that the binding of SAP to apoptotic cells might activate complement. Similarly, Elkon and colleagues reported the calcium-dependent binding of CRP to the surface of apoptotic cells during this time [353], and showed that CRP binding enhanced opsonisation and phagocytosis of apoptotic cells by macrophages. We were interested to determine whether there is competition between the pentraxin proteins, SAP and CRP, for ligands on the surface of apoptotic cells and have robustly demonstrated that this is not the case.

We were able to show that total SAP deficiency in the C57BL/6 mouse enhanced the immunogenicity of injected syngeneic apoptotic thymocytes, and was associated with significant glomerulonephritis after thymocyte injection in female mice. Furthermore, the autoimmunity associated with injecting apoptotic cells persisted and tended to increase in SAP-/- mice (until they were killed, 8 weeks after the first injection) but was usually transient, as reported by Elkon's group [278], in wild-type mice. ANAs were not induced by injection of syngeneic apoptotic thymocytes into SAP-/- 129/Sv mice, further corroborating our findings of absent spontaneous autoimmunity in SAP deficient mice of this strain. Similarly, the induction of comparable autoimmunity between SAP-/- and SAP-/-, hSAP Tg C57BL/6 mice following injection of syngeneic apoptotic thymocytes, which differed significantly from that induced in their wild-type counterparts, support our findings of spontaneous autoimmunity in SAP-/-, hSAP Tg C57BL/6 mice.

The present studies of macrophage ingestion of apoptotic cells, performed *in vivo* using thymocytes injected intraperitoneally and *in vitro* using a human system, failed to show any influence of SAP on macrophage binding and/or ingestion. However, in these preliminary studies the dynamic process of binding and/or ingestion of apoptotic cells by macrophages was only determined at a single time point in both models. Conflicting observations of increased macrophage ingestion of apoptotic Jurkhat cells in the presence of SAP have recently been reported by Bijl *et al*, using an almost identical experimental protocol [354]. However such findings of opsonisation by SAP contrast sharply with previous work from this laboratory showing that SAP is a potent anti-opsonin for bacteria being phagocytosed by neutrophils [69]. Further work is clearly necessary to resolve these issues.

Current evidence suggests that the process of macrophage ingestion of cells dying by apoptosis normally induces expression of anti-inflammatory cytokines [322,350,355]; in contrast, cells dying by necrosis are thought to induce pro-inflammatory cytokines which may promote maturation of dendritic cells and lead to efficient antigen presentation and an immune response [322,350,355]. Apoptotic cells are believed to be the source of the immunogens in SLE, and the pentraxin proteins, which are known to bind to these immunogens [60,61], have been shown here to bind to their surface. SAP deficiency in the C57BL/6 mouse was shown to enhance the immunogenicity of administered apoptotic mouse thymocytes, and pentraxins in general may play an important role in regulating apoptotic cell immunogenicity *in vivo*. Although we were unable to demonstrate any direct influence of SAP on macrophage ingestion of

apoptotic cells, these were only preliminary studies and further investigation of the mechanisms by which pentraxins affect apoptotic cell immunogenicity are warranted.

Chapter 6 – General Conclusions

ROLE OF SAP IN MOUSE ANTINUCLEAR AUTOIMMUNITY

Despite the high degree of evolutionary conservation of the pentraxin proteins SAP and CRP, implying physiological roles with significant survival value, their functions remain unknown.

The creation of SAP knockout mice enabled the participation of SAP in amyloid pathogenesis to be directly demonstrated [208] and SAP has thus become a legitimate target for therapy in systemic amyloidosis. Indeed, an SAP inhibitor developed in collaboration with F Hoffmann-La Roche Ltd is currently undergoing therapeutic trials in patients with amyloidosis [212]. Although SAP knockout mice appeared initially to develop and breed normally, a cohort of [129/Sv x C57BL/6]F_2 mice were noted to spontaneously develop a phenotype resembling human SLE with antinuclear autoimmunity and glomerulonephritis [232]. The binding of pentraxins to nuclear constituents had previously been demonstrated [60-62,299], and it had been postulated some years earlier that pentraxins might have a role in handling chromatin in order to prevent autoimmunisation.

The studies presented here of the spontaneous lupus-like phenotype in pure-line C57BL/6 SAP-/- mice suggest that mouse SAP has an important physiological role, inhibiting the formation of pathogenic autoantibodies against chromatin and DNA.

The dependence of autoimmunity upon mouse strain and its absence in a cohort of inbred 129/Sv SAP-/- mice, highlights the genetic complexity of the lupus diathesis, but corroborates work in mouse models of human SLE in which the "full-blown" SLE phenotype has been shown to depend upon complex genetic interactions between several disease susceptibility loci [215].

Following our observations of spontaneous autoimmunity in C57BL/6 SAP-/- mice we were unable to completely exclude the remote possibility that the phenotype was caused, not by deletion of the SAP gene itself, but by an unknown 129/Sv gene on chromosome 1 linked to the SAP null allele conferring lupus susceptibility when expressed in the C57BL/6 background. In order to address this issue, SAP-/- C57BL/6 mice transgenic for human SAP were developed. Mice expressing the human SAP transgene were compared to their littermate controls in whom the human SAP transgene was not expressed. Interestingly, and perhaps contrary to our initial expectations, the spontaneous autoimmune phenotype was not abrogated by expression of human SAP. On reflection however, it would be surprising if human and mouse SAP functioned identically in the mouse particularly given their different binding affinities *in vitro* [63], and the persistence of autoimmunity in human SAP transgenic mice lacking mouse SAP does not exclude mouse SAP deficiency as the cause of the lupus phenotype.

In order to definitively answer the question of whether deficiency of mouse SAP in C57BL/6 mice is responsible for the lupus-like phenotype, we are currently

investigating transgenic reconstitution of SAP-/- mice with mouse SAP. In a collaborative project with Dr EK Wakeland from the University of Texas, USA, he has introduced extra copies of the mouse SAP gene into a cohort of wild-type C57BL/6 mice and we plan to cross these with our SAP-/- C57BL/6 mice and determine whether expression of mouse SAP (from a chromosome other than chromosome 1) on the C57BL/6 SAP-/- background abrogates the autoimmune phenotype.

APOPTOTIC CELLS, AUTOIMMUNITY AND PENTRAXINS

Apoptosis, a term coined by Currie and colleagues in 1972 [356] describes a common type of programmed cell death and is an essential part of life for any multicellular organism. Indeed, the way in which most cells die is conserved from worm to mammal [357,358].

Apoptotic cells were first proposed to be the source of immunogens in SLE following the observation by Casciola-Rosen and colleagues that the most characteristic "intracellular" autoantigens were present in blebs on the apoptotic cell surface [277]. Some years later, the hypothesis that apoptotic cells are the source of the immunogens in SLE is now supported by considerable experimental evidence [238,239,278,280,281]. However, the exact relationship between nuclear constituents, autoimmunity and apoptotic cells remains unknown.

Certain groups focused on modification of the targets of systemic immune responses during apoptosis thus revealing so called "cryptic epitopes" to which the immune system had not previously achieved tolerance. Many antigens targeted by autoantibodies in systemic autoimmune disease undergo structural modifications during apoptosis including proteolysis and hyperphosphorylation [348,359]. The activation of caspases is the central effector mechanism in apoptosis [360]. The observation that PARP [361] and U1-70 kD [362], two SLE-associated protein autoantigens, were cleaved by caspases during apoptosis led several groups to use ANAs in the systematic identification of other autoantigens cleaved during the cell death process. Casciola-Rosen and colleagues and Casiano and colleagues separately identified subsets of intracellular autoantigens that were cleaved during apoptosis [348,359,363]. Notably however, many autoantigens frequently targeted by autoantibodies in systemic autoimmune diseases remain intact during apoptosis [348,359,364]. Progression from apoptosis to secondary necrosis was associated with additional proteolysis of specific intracellular autoantigens that are cleaved during apoptosis [365]. These results suggest that in the absence of phagocytosis progression of cell apoptosis to secondary necrosis involves additional degradation of specific autoantigens, with potential immunostimulatory consequences. However, there is scant evidence that exposure of the immune system to modified forms of apoptotic self-antigens are sufficient to elicit autoantibodies. Under what circumstances then, does apoptotic cell material provoke immune responses? Sauter and colleagues suggested that uptake of apoptotic material by dendritic cells may lead to an immune response only if followed by a maturation signal provided under a

pro-inflammatory context [366]. According to this notion, apoptosis associated with normal cellular turnover induces dendritic cells or other phagocytic cells to produce anti-inflammatory molecules and cross present apoptotic cell antigens to the immune system, leading to induction of peripheral tolerance. Induction of peripheral tolerance arises due to the fact that in the absence of pro-inflammatory signals, immature dendritic cells and other professional phagocytes display low levels of MHC class I, class II and costimulatory molecules thus inducing self tolerance rather than initiating immune responses [367]. Evidence for this hypothesis has come from a number of studies in which pro-inflammatory conditions, such as an infection, were associated with dendritic cell maturation, up-regulation of MHC molecules and co-stimulatory signals, presentation of self-peptides derived from dying cells (as well as foreign peptides from the infectious agent) and efficient activation of autoantigen-specific T and B cells [368-370].

Our discovery of enhanced immunogenicity of intravenously administered syngeneic apoptotic thymocytes into SAP-/- C57BL/6 mice compared to their wild-type counterparts, suggested a role for mouse SAP in handling apoptotic cells *in vivo*. Confirmation that the pentraxins, SAP and CRP, bind to the surface of apoptotic cells suggests that the mechanism by which SAP inhibits autoimmunity in the mouse is either by affecting uptake of or response to apoptotic cells by macrophages or other phagocytic cells thus influencing apoptotic cell immunogenicity, or by binding and regulating the clearance and/or degradation of chromatin and nucleosomes released from dying cells *in vivo*, or both.

The final phase of apoptosis, the programme of cell deletion *in vivo*, is swift and safe phagocytosis of intact unwanted cells. Although ignored for many years, the removal of apoptotic cells by macrophages is now thought to define the meaning of cell death in higher organisms and critically regulate immune responses [371]. Cells undergoing apoptosis can display a number of "eat-me" signals. Some, such as exposure on the outer membrane leaflet of phosphatidylserine, which is usually restricted to the inner membrane leaflet, are relatively well characterised [335]. Others are more poorly defined, such as sites that bind adhesive bridging molecules present in extracellular fluid. These include C1q [279,331], β_2 glycoprotein I (β_2GPI) [372] and thrombospondin [373]. A role for C1q *in vivo* was confirmed by studies demonstrating defective clearance of apoptotic cells in deletion-mutant mice [279,331]. It is interesting that a cohort of C1q deficient [129/Sv x C57BL/6]F_2 mice were discovered to develop a spontaneous lupus-like phenotype [331] similar to that reported from our laboratory in SAP deficient [129/Sv x C57BL/6]F_2 animals [232], but that C1q deficiency on a pure C57BL/6 background was insufficient to cause the lupus phenotype (verbal communication, M Botto), markedly contrasting with the autoimmunity presented here in SAP deficient pure-line C57BL/6 mice.

The potential bridging role of the abundant β_2GPI was demonstrated by Schroit and colleagues in studies in which macrophages were found to bind β_2GPI only after it was complexed with phosphatidylserine (PS) [372]. Chemical modification of cysteines abolished β_2GPI-dependent PS uptake by inhibiting the binding of PS-β_2GPI complex to macrophages. Antibodies to β_2GPI inhibited binding of the

complex to macrophages indicating that recognition was mediated by β_2GPI and not by the lipid. Furthermore, antibodies to putative macrophage PS receptors such as CD36, CD68 and CD14 did not inhibit uptake of the PS-β_2GPI complex by macrophages [372]. However, there is evidence that phagocyte scavenger receptors such as CD68 recognise apoptotic cell surface sites that can be masked by antibodies against oxidised low density lipoproteins (LDL) [374]. Interestingly, and of relevance to the work presented in this thesis, Chang and colleagues recently reported that CRP binds to both apoptotic cells and oxidised LDL through recognition of a common ligand [375].

Although we are aware of a number of mammalian phagocyte receptors that mediate the engulfment of dying cells, we do not yet know what contribution is made to the clearance task by each molecule. There is evidence that macrophages might "tether" dying cells by using phagocyte surface CD14 or β_2 integrins before engaging receptors that drive phagocytosis. The relative importance of the dynamic reorganisation of the phagocyte membrane which is dependent on lipid fluxes similar to those occurring in apoptotic cells themselves also needs clarification. Our current understanding of the molecular mechanisms mediating apoptotic cell clearance thus remains poor. The apparent redundancy of many of the mechanisms mentioned is consistent with the importance for health of safely clearing apoptotic cells but might equally reflect the exquisite control of phagocyte responses which may modulate inflammation. The potential significance programmed into these molecular mechanisms was suggested by a seminal *in vitro* experiment.

Macrophages are crucial for both clearance of apoptotic cells generated in injured tissue and for host defence against infection by bacteria or protozoa. Normally the ingestion of particles of similar size to these invaders triggers macrophages to secrete molecular mediators capable of initiating protective but potentially injurious inflammatory responses. The ingestion of large numbers of apoptotic cells failed to elicit this macrophage release of pro-inflammatory mediators. If, however, experimental conditions were deliberately changed so that recognition mechanisms for "quiet clearance" were replaced by bridging immunoglobulin and macrophage Fc receptors, macrophages released pro-inflammatory mediators [376].

Although inflammatory responses are vital for host defence against infection, when persistent they underlie important diseases such as rheumatoid arthritis. Dangerous immune cells can be quietly cleared from inflamed sites by undergoing apoptosis and being engulfed by macrophages promoting the resolution of acute inflammation [373]. Indeed, uptake of apoptotic cells actively suppresses the secretion from activated macrophages of inflammatory mediators such as tumour necrosis factor-α (TNF-α) [322,350]. How is anti-inflammatory meaning conferred on phagocytic clearance of apoptotic cells? "Resetting" of activated macrophages can be mimicked by the ligation of macrophage receptors mediating the engulfment of apoptotic cells, notably CD36 and its bridging ligand thrombospondin [350]. Indeed, receptor-triggered release of the anti-inflammatory and immunosuppressive cytokine transforming growth factor β1 (TGF-β1) by macrophages ingesting apoptotic cells might be crucial in mediating the suppression of macrophage-directed

inflammation [322,350,377]. Macrophages are nevertheless activated to secrete pro-inflammatory mediators by the ingestion of white blood cells undergoing secondary necrosis after apoptosis, but not by intact apoptotic cells [378]. Therefore, whether clearance has an anti- or pro-inflammatory meaning might be determined by the state of the dying cell, the phagocyte receptors engaged and the downstream signalling pathways activated.

The anti-inflammatory action of phagocyte clearance of apoptotic cells might also be perturbed in disease processes. For example, anti-phospholipid antibodies that recognise and bind to PS exposed by apoptotic cells can be found in patients with SLE. Such autoantibodies can coat apoptotic cells so that they are bound by macrophage Fc receptors with resultant promotion of TNF-α release rather than its suppression [379].

Studies presented in this work compared macrophage uptake of apoptotic cells in the presence and absence of SAP and were performed in a mouse model *in vivo* and in a human system *in vitro*. Although these were only preliminary studies, we were unable to determine any influence of SAP on macrophage ingestion of apoptotic cells in either system. These results conflict with the observations of Bijl and colleagues who, using an almost identical experimental protocol, recently reported increased macrophage ingestion of apoptotic Jurkhat cells in the presence of SAP [354]. Bijl's findings also conflict with previous work from our laboratory which

showed SAP to be a potent anti-opsonin. However, these *in vitro* experiments were in the context of neutrophil phagocytosis of bacteria [69].

Our studies do not exclude the possibility that SAP may influence macrophage ingestion of apoptotic cells and hence their immunogenicity in a more subtle, but nevertheless important manner. Indeed, binding of CRP to apoptotic cells *in vitro* was shown by Elkon and colleagues to amplify classical pathway activation of complement on the surface of the dying cell but paradoxically attenuate deposition of the membrane attack complex (MAC) and inhibit cell lysis [353]. However, CRP enhanced opsonisation of apoptotic cells by macrophages associated with expression of the anti-inflammatory cytokine transforming growth factor β (TGF-β). Evidence exists therefore, that coating of apoptotic cells by CRP promotes an anti-inflammatory cytokine profile from ingesting macrophages. The absence of pentraxin proteins bound to the surface of apoptotic cells might thus lead to release of pro-inflammatory cytokine mediators from ingesting macrophages, similar to those expressed following ingestion of necrotic cells.

NUCLEOSOMES, AUTOIMMUNITY AND PENTRAXINS

The plasma of patients with SLE contains increased levels of nucleosomal-like DNA that closely resemble the characteristic oligonucleosomal ladder generated during apoptosis [324]. This observation directly implicated apoptosis as the possible source of immunostimulatory nucleosomes and was consistent with studies pointing to nucleosomes as the *in vivo* immunogens driving the anti-DNA and anti-histone

immune responses in SLE [269-272]. This notion was further strengthened by reports that spontaneous apoptosis of cultured mouse lymphocytes was associated with the release of immunostimulatory nucleosomes [380-382]. In agreement with these studies, apoptosis of lymphocytes from SLE patients *in vitro* was shown to be accompanied by the release of nucleosomes into the extracellular milieu [280]. Furthermore, *in vivo* studies showed that serum nucleosomal DNA naturally occurs in autoimmune mice and correlates with apoptosis in the thymus [383]. In SLE patients, the plasma levels of nucleosomes were shown to correlate with serum antinucleosome antibody titres [324].

Serum amyloid P component (SAP) is the major calcium-dependent DNA and chromatin binding protein of the plasma [60]. SAP was shown some time ago in our laboratory to specifically displace H1 histone and solubilise native long chromatin and it was suggested that its major physiological role might be to enhance non-immunogenic clearance of nuclear material, including chromatin, which is released from such cells [61].

Studies presented in this work comparing plasma clearance of intravenously administered nucleosomes and chromatin in the presence and absence of SAP, consistently showed that the presence of SAP enhanced their rate of clearance. Furthermore, studies using the trapped catabolism method, showed robustly and for the first time that nucleosome catabolism occurs largely in the liver and kidneys, but that in the absence of SAP, early uptake of nucleosomes into the spleen is increased.

Thus, SAP leads to rapid removal of nucleosomes and chromatin from the plasma and may have a pivotal role in preventing dose-related immunogenic processing of nuclear constituents in the spleen.

SUMMARY

The studies presented in this thesis suggest that deficiency of the plasma pentraxin proteins SAP and CRP contribute to the lupus diathesis. The finding of striking spontaneous antinuclear autoimmunity in SAP deficient pure-line C57BL/6 mice coupled with the confirmation of binding of SAP and CRP to apoptotic cells and the previously reported binding of SAP to chromatin and DNA suggest a physiological role for pentraxins in handling of chromatin *in vivo*.

Current evidence strongly implicates defective apoptotic cell processing and clearance in the development of systemic autoimmunity. Binding of CRP to the apoptotic cell was recently shown to amplify complement classical pathway activation on its surface but protected the dying cell from assembly of the terminal complement components and lysis. CRP also enhanced opsonisation of apoptotic cells by phagocytes and was associated with expression of anti-inflammatory cytokines. Studies of SAP binding to apoptotic cells *in vitro* and *in vivo* were not shown in this work to influence macrophage uptake of apoptotic cells, although recently published work conflicts with our findings and suggests that binding of SAP, like CRP, results in opsonisation of apoptotic cells. There is considerable experimental evidence that defective apoptotic cell clearance is associated with

increased immunogenicity and this may be due to loss of membrane integrity, and exposure of increasingly degraded autoantigens with consequent presentation of cryptic epitopes as uncleared apoptotic cells progress to secondary necrosis. In addition, the milieu in which phagocytic cells engulf apoptotic cells appears to play a pivotal role in control of immune responses. In the presence of pro-inflammatory conditions such as an infection, dendritic cells and professional phagocytes receive a maturation signal and efficient activation of autoantigen-specific T and B cells ensues. In contrast, apoptosis associated with normal cellular turnover induces engulfment by immature dendritic cells and phagocytes which produce anti-inflammatory molecules leading to induction of peripheral tolerance.

It is likely that the loss of membrane integrity associated with progression of cells from early apoptosis to secondary necrosis is associated with increasing release into the circulation of nuclear material contained within apoptotic cell surface blebs such as nucleosome core particles and DNA. It is known that the plasma of patients with the prototypic systemic autoimmune disease SLE contains increased levels of nucleosomal-like DNA that closely resembles the characteristic oligonucleosomal ladder generated during apoptosis. SAP is the single plasma protein that shows calcium dependent binding to nucleosomal DNA and chromatin and *in vivo* experiments presented in this thesis suggest that an alternative mechanism whereby plasma pentraxins, in particular SAP, may influence immunogenicity of nucleosomes is by binding to them and enhancing their rate of clearance from the plasma. Furthermore, *in vivo* studies consistently demonstrated that SAP deficiency

was associated with increased early uptake of nucleosomes and chromatin into the spleen. It is attractive to postulate that SAP deficiency, perhaps in the setting of dysregulated apoptosis, might lead to presentation of a critical mass of nucleosomal autoantigens to the immune cells of the spleen thereby enhancing antigen-specific T cell activation.

Despite being unable, at present, to completely exclude the remote possibility that the lupus-like phenotype in SAP-/- C57BL/6 mice is SAP independent, we believe this to be unlikely. SAP is the only major calcium dependent DNA and chromatin binding protein of the plasma and we have shown in this work that its binding to chromatin and nucleosomes affects their rate of plasma clearance and site of catabolism. Consistent with this notion, work published from other groups within the last few years has suggested that the interaction between pentraxins and apoptotic cells has immunomodulatory consequences.

Finally, no deficiency state or polymorphism at the amino acid level has been identified in the human SAP or CRP genes. A CRP intron polymorphism however, has been shown to influence basal levels of CRP [384] and a CRP promoter polymorphism has been shown to be associated with type II diabetes mellitus susceptibility in Pima Indians [385]. There is considerable evidence that basal CRP concentration is important in cardiovascular disease risk [172,386,387]. A deficient CRP response to autologous tissue damage in patients with SLE and the finding of a lupus-like phenotype in SAP deficient mice raise the possibility that CRP responder

status or basal CRP and/or SAP concentration may contribute to the genetic predisposition to human SLE. Further work is required to determine whether patients with SLE and their relatives have defects in the structure, regulation, metabolism or function of SAP and/or CRP. Similarly, further work is required to definitively answer whether plasma pentraxins, when they bind to apoptotic cells and their immunogenic contents, opsonise or behave as anti-opsonins and determine the exact downstream immunomodulatory effects.

Appendix 1 – Proposals For Future Research

MOUSE STUDIES

Our observations of spontaneous antinuclear autoimmunity in pure line C57BL/6 SAP-/- mice coupled with extensive *in vitro* evidence of SAP binding to apoptotic cells, chromatin, histones and DNA provide strong evidence that SAP deficiency is responsible for the autoimmune phenotype. However, we are unable from these studies alone to categorically rule out the remote possibility that the observed autoimmune phenotype is not due to SAP deficiency itself but instead due to the interaction between an unknown 129/Sv gene on chromosome 1 linked to the SAP null allele and the remainder of the C57BL/6 genome.

Studies to address this critical issue are already underway. The aim is to introduce extra copies of the mouse SAP gene into a cohort of wild-type C57BL/6 mice. Mice into which the extra SAP gene has been successfully introduced and is expressed will then be crossed with our SAP-/- C57BL/6 mice. We will then be able to determine whether expression of mouse SAP (from a chromosome other than chromosome 1) is sufficient to abrogate the autoimmune phenotype in our C57BL/6 SAP-/- mice.

APOPTOTIC CELLS, AUTOIMMUNITY AND PENTRAXINS

Several publications during the last few years have corroborated the findings presented in this thesis of *in vitro* binding of the pentraxins, SAP and CRP, to apoptotic cells. Although binding of CRP has been shown to opsonise apoptotic cells and enhance macrophage uptake *in vitro*, further studies are required to confirm these findings. Similarly, further *in vitro* studies are required to determine the immunomodulatory effects of SAP binding to apoptotic cells both in terms of macrophage uptake, where our results conflict with the findings of others, and in terms of macrophage cytokine profiles where, thus far, no work has been published.

In this work, we were unable to detect any effect of SAP on macrophage uptake of apoptotic mouse thymocytes *in vivo*. However, further work of a similar nature is required in which both uptake by stimulated and unstimulated macrophages and their immunologic consequences is examined at different time points. The considerable challenge to any investigator attempting to determine such effects is how to examine *in vitro* what is essentially a dynamic process *in vivo*.

NUCLEOSOMES, AUTOIMMUNITY AND PENTRAXINS

Using the trapped catabolism method we were able to determine that the liver and kidneys are the major sites of nucleosome catabolism. SAP was also shown to accelerate clearance of nucleosomes and chromatin from the plasma and divert nucleosome uptake away from the spleen. Further experiments are required to

determine the downstream effects of SAP deficiency in terms of antigen processing. It would be particularly interesting to compare intravenous injection of an autologous lupus antigen in the presence and absence of SAP and determine whether SAP deficiency resulted in an increase in the antigen-specific splenic T cell repertoire or enhanced activation of antigen-specific T cells in the spleen.

One of the experimental difficulties encountered during our *in vivo* nucleosome clearance experiments was the degradation over time of the starting material. Unfortunately, further experiments of this nature are likely to be hampered by the batch to batch variation of nucleosome preparations which is extremely difficult to overcome.

HUMAN STUDIES

The deficient CRP response to autologous tissue damage during SLE flares and the key role of pentraxins in animal models of lupus suggest that SAP and/or CRP may be important candidate genes in SLE. Moreover linkage analysis has demonstrated important SLE susceptibility loci on chromosome 1 and the pentraxin genes are within certain of these candidate linkage regions. Further family studies will continue to narrow the respective disease susceptibility regions and should eventually point to relatively few candidate genes contained within each locus.

Although recently established, the genetic component to baseline CRP concentration is not fully understood. Whether there is a genetic component to CRP

responsiveness remains unknown. If these genetic components are clearly defined in the near future as seems likely, it will be interesting to determine whether they behave as SLE susceptibility factors. Similar studies may reveal a genetic component to baseline SAP concentration which may uncover a hitherto unknown relationship with susceptibility to SLE.

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